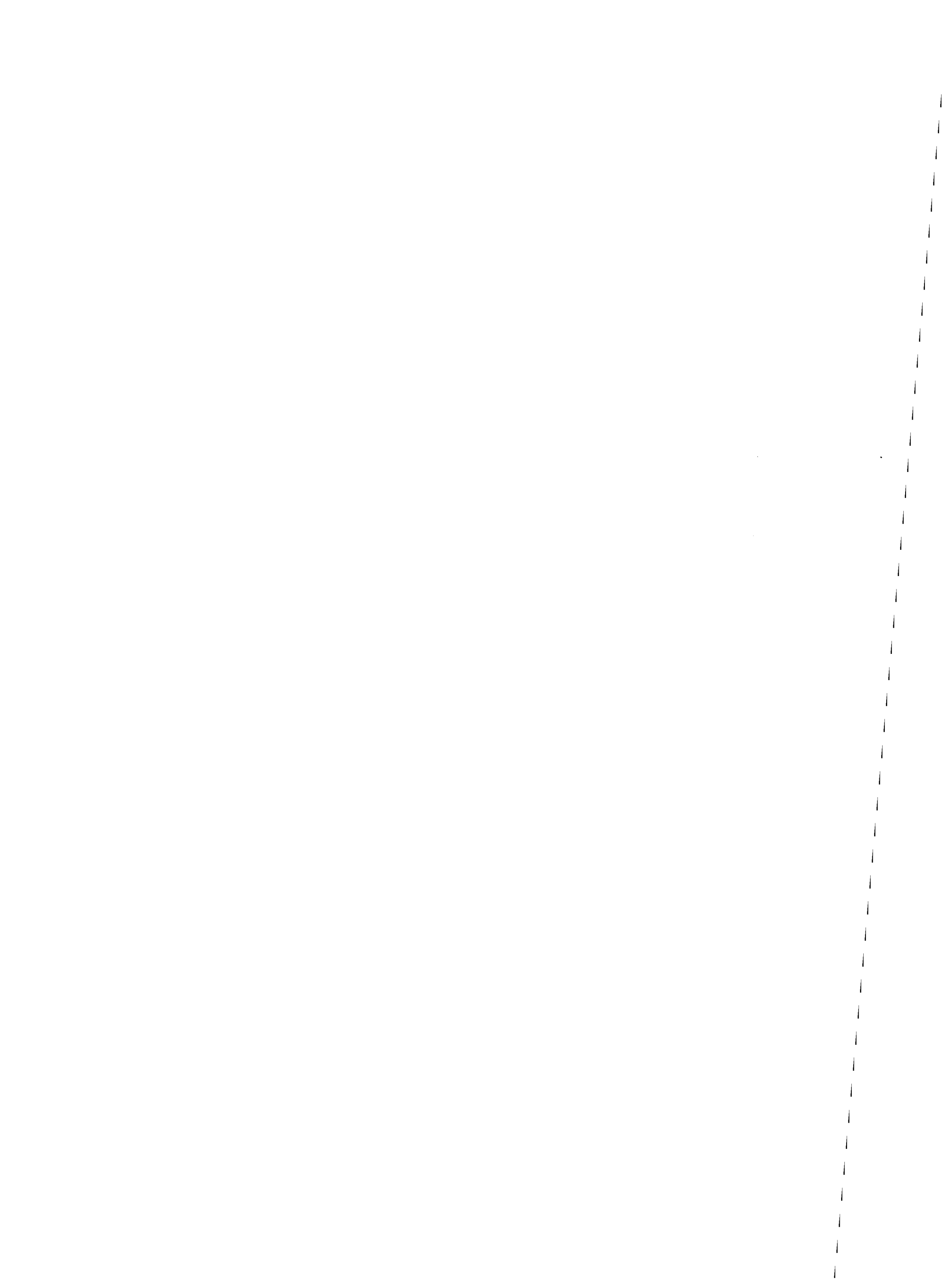


**“Aspects of the Biology of a House Sparrow
(*Passer domesticus*) Colony”**

by

Jon Wetton, B.Sc.

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ABSTRACT

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ABSTRACT

Morphological, biochemical and minisatellite DNA variation was investigated at the colour ringed Brackenhurst House Sparrow population. Measurements and blood samples were collected from 584 nestlings and 692 other birds between 1985 and 1989. Six loci (6PGD, IDH, PEPD2, PEPD3, PEPT and transferrin) which had been the subject of a previous report (Burke, 1984) were investigated by starch gel electrophoresis. All followed Mendelian inheritance patterns, were in Hardy Weinberg equilibria and displayed temporal stability in allele frequencies. No evidence was found of the previously reported segregation distortion at PEPD3 and transferrin but artefact bands were encountered when scoring the latter.

Family groups identified by observing colour ringed adults during feeding visits were examined using both enzyme and minisatellite DNA markers. Z chromosome linkage of several fingerprint bands was implicated, though most segregate independently. The probability of detecting an extra-pair fertilization was estimated as 0.5454 using starch gels and 0.9998 by fingerprinting. 51 out of 420 nestlings from 144 broods possessed several bands absent from the attendants' fingerprints. All nestlings with multiple mismatches shared many bands with the attendant female but a number consistent with band sharing between unrelateds with the male, i.e. non-parentage, was the result of cuckoldry. 24% of broods and 37% of males were affected. A correlation between the presence of extra-pair offspring and poor hatching success was noted. Cuckoldry was twice as successful in broods which contained infertile eggs.

Metric variation was examined in the confirmed families. Significant heritabilities were demonstrated for weight, tarsus and tail length but environmentally induced variance was considerable. Yearlings were smaller than full adults in plumage length. This may be due to levels of protein reserves at critical growth periods. Some

evidence of assortative mating for tail length was found which was unrelated to age associated changes.

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CHAPTER 1

INTRODUCTION

Field based studies integrating the disciplines of genetics, ecology and behaviour have become more commonplace as advances in molecular biology have uncovered genetic markers which can be applied to problems in demographic genetics and behavioural ecology. Current areas of interest in population biology include individual lifetime reproductive success, the genetic structure of populations and estimating the heritabilities of quantitative traits all of which require a precise knowledge of the genetic relatedness between individuals. However, observations of natural populations have shown that even in supposedly monogamous species the positive identification of parents and offspring can be in doubt (McKinney *et al.*, 1984).

This thesis will examine the population biology of a colour-ringed House Sparrow (*Passer domesticus*) colony. Family groupings identified by observation of the attendant adults at the nestbox will be used to quantify morphological, biochemical and DNA sequence variations, and to describe their mode of inheritance. Anomalies in the inheritance of these traits may arise when the parents have been incorrectly assigned. The frequency with which this occurs through individual birds pursuing reproductive strategies other than strict monogamy will be determined. By firstly establishing the inheritance patterns of a variety of genetic markers amongst individuals of known parentage, their reliability and power for detecting cases of non-parentage can be determined. Having done this the factors influencing the extent and success of alternatives to monogamy can be identified.

Genetic Variation

Two opposing views on the extent of genetic polymorphism were current before the application of molecular biology to the study of genetic variation. The “Classical” hypothesis championed by Muller (1962) was strongly influenced by Morgan’s pioneering laboratory studies of *Drosophila* mutants. These mutants were used because of the drastic effect that the genotype had on the phenotype enabling them to be easily identified. However, carriers of mutant alleles were usually less fit than wild type individuals particularly when homozygous. From these observations it was concluded that the vast majority of genetic variation which arises through mutation is deleterious and is rapidly eliminated by purifying selection whilst those rare mutations which conferred an advantage on their carriers are swept to fixation by directional selection. This led to the prediction that very few loci would be heterozygous. As recently as 1966 Muller and Kaplan in a defence of the classical view predicted that a human individual is unlikely to be heterozygous at more than 80 loci and probably at as few as eight.

The alternative hypothesis (e.g. Wallace, 1958) predicted that individuals would be heterozygous at the vast majority of loci with variation being maintained by some form of balancing selection. According to this model there is no single wild type allele but a range of alleles at most loci which attain moderate frequencies. Together they can form a variety of genotypes that are suitable in the habitats encountered by the population. This view gained much support from the work of the ecological geneticists (Ford, 1975). They found that different morphs could be exposed to selective forces which favoured the maintenance of polymorphisms. No single genotype conferred superior fitness but many different genotypes influenced by epistatic interactions between loci and the environment produced phenotypes that were viable in varied environments.

Morphological Variation

Some of the clearest examples of the selective maintenance of polymorphisms were found in the Lepidoptera and snails. The rapid increase in the frequency of melanistic morphs of butterflies and moths closely paralleled the darkening of their natural substrates by industrial pollution, and their subsequent decline has accompanied the introduction of pollution controls (Kettlewell, 1973). Visual predation is the major selective agent in this case and is also largely responsible for the long established polymorphisms of *Cepaea nemoralis* (Cain and Sheppard, 1954). In this species selection has acted simultaneously on several loci which control shell banding number and intensity, and background colour, to produce coadapted complexes conferring phenotypes which camouflage the snails in a variety of habitats (Cain *et al.*, 1960). However, visual polymorphisms under the genetic control of one or a few loci with clearly defined effects are rare in natural situations and have been siezed upon by geneticists because of the ease of their interpretation rather than the intrinsic interest of the species itself.

Most morphological variation occurs in the form of a continuum without clearly demarcated morphs, the differences between individuals being quantitative rather than qualitative. The methods for analysing quantitative traits are an extension of Mendelian genetics. However, the role of a particular locus cannot be easily determined because the heritable component originates from many loci each affecting the trait to a varying degree and each having its effects modified by interactions with other loci and the environment such that the genotypic classes are blurred into a continuously graded series.

A heritable basis for many quantitative traits had long been known through the tendency of 'like to beget like'. This formed the principle of selective breeding which had been applied to crops and livestock for thousands of years. Darwin had noted the

ease with which man had been able to modify the characteristics of other organisms to his own requirements and used this as a major line of evidence in proposing the widespread potential for natural selection (Darwin, 1859). Artificial selection has been successfully applied to at least 51 traits in *Drosophila melanogaster* alone, including body and wing size, number of abdominal bristles, resistance to DDT, rate of development, fecundity and behavioural traits such as geotaxis and phototaxis (Dobzhansky *et al.*, 1977). That very few studies have failed to select for a particular characteristic suggest that heritable quantitative variation is almost ubiquitous, although it must be borne in mind that unsuccessful experiments are seldom reported.

The presence of morphological variation within a species does not in itself imply the existence of genetic variation. James (1983) transplanted nestling Red-winged Blackbirds (*Agelaius phoeniceus*) between the ends of a body and beak size cline in N. America and found that they grew to resemble their foster parents more than their biological parents. Experiments of this nature demonstrate that the influence of the environment can outweigh the effect of the genotype. This problem has hampered the genetic analysis of quantitative traits in wild animals, most work being carried out with domesticated or laboratory strains under closely controlled conditions where individuals of known relatedness can be raised under similar circumstances to determine the extent to which shared genes lead to morphological similarity.

In recent years the increasing number of long term field studies of marked populations has allowed similar investigations to be attempted under natural conditions. These have largely been restricted to birds because they are relatively easy to capture, mark and subsequently identify. They can also be induced to raise their young in nestboxes which allow easy access for the experimenter and provide similar natal environments for all nestlings during much of the critical growth period. Significant heritabilities have been demonstrated for bill characteristics, weight, wing and tarsus

length in several passerine species by comparison of parents and offspring (refs in Boag and van Noordwijk, 1987).

By quantifying the heritable proportion of observed metric variation workers can estimate the strength of selection on morphological traits from the difference between pre- and post-selection generations. Some of the highest selection coefficients yet measured have been associated with quantitative traits. The change in beak size of the Galapagos Ground Finch, *Geospiza fortis*, following the El Niño induced drought of 1977 resulted from the birds' dependence on the large hard seeds of caltrop (*Tribulus cistoides*) and Opuntia which formed virtually the only food source. An individual's ability to handle seeds was determined by its beak size. Therefore, those with the largest bills survived the famine to produce the next generation. The increase in beak depth of 4% combined with the heritability estimate (0.79) derived from parent offspring comparisons suggested a selection differential as large as 0.6 had been applied by the drought (Grant, 1986; Boag and Grant, 1981).

Despite an obvious genetic basis to many metric traits the difficulty in estimating the number of loci involved let alone the precise effects of individual alleles meant that little evidence was available in the debate on the proportion of heterozygous loci. This situation changed with the development of starch gel electrophoresis during the 1960's.

Protein polymorphisms

Starch gel electrophoresis will detect changes in the base sequence of a structural locus if they result in the substitution of an amino acid which alters the charge, size or shape of the final protein product. Electrophoresis separates the different allelic forms, known as allozymes, on the basis of these characteristics. By careful choice of gel concentrations, pH, applied voltage and ionic concentration the

differences in migration distances can be maximized. The final position of each allozyme can then be determined by direct staining or through reactions linked to the enzyme's function which yield a coloured product. Many loci can be scored in a single individual and due to the technique's simplicity, large samples can be surveyed.

The technique was first applied to large numbers of loci in humans and *Drosophila pseudoobscura* (Harris, 1966; Hubby and Lewontin, 1966) . These, and subsequent investigations in a variety of species, revealed that many loci are polymorphic with average heterozygosities amongst soluble protein loci of the order of 10%. However, the technique under-estimates the true frequency of variation as only 25% of nucleotide substitutions are detectable. Many bring about no change in the amino acid due to degeneracy of the genetic code (King and Jukes, 1969) whilst some amino acid substitutions leave the charge of the protein unchanged. More varied electrophoretic techniques (Aquadro and Avise, 1982) and thermal stability studies (Singh, Hubby and Throckmorton 1975) have revealed that a single band on a gel (an electromorph) may represent more than one allele with identical charge but differing in biochemical properties.

Since the majority of proteins studied are soluble enzymes they form a biased sample of structural loci and may not be truly representative of other loci. Feather proteins are variable between species but tend to be monomorphic within them (Knox, 1980), whereas C6, a component of the complement system, is multi-allelic and highly heterozygous in birds and mammals (Whitehouse, 1979). The application of 2-D gel electrophoresis to a wider range of loci seemed to indicate a lower heterozygosity ($\bar{H} = 0.02 - 0.04$) (e.g. Racine and Langley, 1980) though doubt has been cast on this finding due to its lower sensitivity as compared with 1-D electrophoresis (McLellan *et al.*, 1983).

While the exact degree of heterozygosity can only be determined by direct inspection of the DNA sequence it had become clear that the amount of variation was too high to be maintained by balancing selection or heterosis at most loci. The genetic load imposed by such a selection regime would be intolerable. This led Kimura to formulate the Neutral Theory of molecular evolution (Kimura, 1983). Kimura and Crow (1964) showed that the expected mean equilibrium level of heterozygosity (\bar{H}) at loci subject to mutation and random genetic drift is given by the relationship

$$\bar{H} = 4N_e\mu / \sqrt{4N_e\mu + 1}$$

where N_e is the effective population size and μ is the mutation rate per locus per generation. They suggested that the majority of changes at the molecular level are selectively neutral and that their fate is determined purely by stochastic processes. The observed levels of heterozygosity are brought about by neutral alleles drifting in frequency. Selection would still operate on both deleterious and advantageous mutants as predicted by the classical theory and so neutralism has been described as the “neo-classical” hypothesis (Lewontin, 1974).

Clearly the proportion of nucleotide substitutions which might be selectively neutral depends on the locus in question. House-keeping enzymes involved in the major biochemical processes such as the Krebs cycle typically exhibit less polymorphism than those which act on external substrates. The former typically work on a specific substrate and changes in secondary structure resulting from an amino acid substitution are liable to influence the binding capacity and reaction kinetics of the enzyme and in some instances the flux through the pathway. These enzymes are often monomorphic within species and quite highly conserved between taxa whilst those that are most diverged between species tend to be heterozygous within them. The enzymes falling in the second class usually act on more than one external substrate, thus an optimal secondary structure is less likely, the weaker constraints on structure and specificity increases the number of mutations that are selectively neutral.

Heterozygosity is generally higher in ectotherms than endotherms. The greater degree of homeostasis in endotherms provides a constant environment in which an optimised allozyme can function. However, the correlation is not clear-cut because endotherms can be stressed by hypo- and hyper-thermy whilst some ectotherms live in environments which are essentially homogeneous and unchanging, e.g. deep sea fish and benthic invertebrates.

The relative importance of selection and stochastic processes in the maintenance of protein polymorphisms has not been resolved by the accumulation of data provided by electrophoresis. Proving selection for a biochemical character is a difficult task as the selective agent and mode of selection have to be determined (Clarke, 1975). The sickle cell haemoglobin polymorphism (Allison, 1964) remains the only fully proven example of the direct selective maintenance of a protein polymorphism. The sickle cell allele greatly reduces fitness in the homozygous form through its poor oxygen binding characteristics and its high turnover. In the heterozygous state the effect is mild but it also confers protection against malarial infection to which homozygotes for the normal allele succumb. Heterozygote advantage is clearly capable of maintaining variation at this locus in the presence of malaria. A further direct association between an allozyme and malarial resistance is likely to be confirmed for Glucose-6-phosphate dehydrogenase (G6PD) (Wills, 1980).

Strong evidence for the selective maintenance of clines in allele frequency has been compiled for the alcohol dehydrogenase (ADH) and α -Glycerophosphate dehydrogenase (α -GPDH) loci in *Drosophila melanogaster* and for lactate dehydrogenase (LDH) in *Fundulus heteroclitus* (Koehn *et al.*, 1983). The allozymes at these loci differ in their temperature dependent catalytic properties and this may explain their geographic distribution.

Although little has been published on the biochemical aspects of avian enzyme polymorphisms, several studies have found evidence for an association between genotype and fitness differences or geographic distribution. Spatial variation has been correlated with habitat differences in the Mute Swan (*Cygnus olor*) and Blue Grouse (*Dendragapus obscurus*). Grouse heterozygous at the Ng locus are much more common in mature forests than young plantations (Redfield, 1973, 1974) whilst a rare LDH allele of the Mute Swan attains unusually high frequencies at two sites in South West England where the birds are colonial rather than territorial as elsewhere, despite a high rate of immigration (Bacon, 1979).

Selection may be responsible for the transferrin polymorphisms of the Ring-necked Pheasant (*Phasianus colchicus*) and Pigeon (*Columba livia*) which appear to be maintained by the greater resistance to infection of eggs laid by heterozygous females. Egg conalbumin, which is encoded by the same locus as transferrin, inhibits the growth of pathogens (Lucotte and Kaminski, 1976; Frelinger, 1971 and 1972).

Reproductive advantages in terms of higher productivity and earlier egg laying are demonstrated by certain LDH and esterase genotypes of the Mute Swan (Bacon, 1979). Differential survival has been noted between genotypes in the Dark-eyed Junco (*Junco hyemalis*). Dominant individuals in flocks tend to be heterozygous for L-leucyl-glycl-glycine peptidase. This genotype is associated with large body size and faster weight gain following food restrictions. Both are important components of survival during food shortage.

Consistent differences in allele frequency at the adenosine deaminase (ADA) locus of the House Sparrow have been found between urban and rural habitats (Cole and Parkin 1986). The enzyme plays an important role in the catabolism of food in the digestive tract and so the difference in frequency may be correlated with exposure to different foodstuffs. More direct evidence for an association between food supply and

genotype is provided by the EST-2 locus of starlings (*Sturnus vulgaris*). Heterozygous birds appear to be unable to digest poultry meal which accumulates until it blocks the oesophagus, killing the bird. In none of these species has it been demonstrated that the phenomena observed are the result of selection acting on the locus under investigation and not on one in tight linkage disequilibria with it. The examples cited above are, however, the exception - most avian polymorphisms are explicable by stochastic processes without resorting to the invocation of selection.

Evan's (1987) review of allozyme studies in birds lists 171 species. In general, they are polymorphic at a similar proportion of loci (\bar{P}) and share similar mean heterozygosities (\bar{H}) with mammals but they are much less variable than invertebrates ($\bar{P} = 0.150$ versus 0.397 and $\bar{H} = 0.047$ cf 0.12). The dichotomy between vertebrates and invertebrates may reflect differences in homeostasis and the larger effective population size of invertebrates.

The majority of studies in Evan's review were aimed at measuring genetic differentiation between populations and species. However, in recent years the technique has been applied more frequently to demographic problems following the pioneering work of Hanken and Sherman (1981) who used polymorphic enzymes as genetic markers in an investigation of the mating system of Belding's Ground Squirrel (*Spermophilus beldingi*).

Avian Mating Systems

A wide variety of social groupings have been recorded in bird species. By far the most common (>90%, Lack 1968) is the monogamous pair but the remainder include virtually all the combinations observed in other vertebrates (references in Sherman, 1983 and Oring, 1982). Some are polygynous (e.g. Red-winged

Blackbirds), lekking (e.g. White-bearded Manakins, *Manacus manacus*) or parasitic (e.g. Cuckoos, *Cuculus canorus*). In others the female regularly mates with several males, e.g. male Galapagos Hawks (*Buteo galapagoensis*) cooperate to raise the young of their single shared mate. Polyandry is common in the phalaropes which show reversed sexual dimorphism, the female being larger and more colourful. Polyandry amongst phalaropes is favoured by the very brief breeding season in high northern latitudes which limits female reproductive success, by preventing them from caring for successive broods. However, the superabundance of food relaxes the constraints on egg laying, allowing females to mate sequentially with several males, leaving them to incubate the eggs alone. Avian mating systems reach their highest complexity in the polygynous/polyandrous system of the Ostrich (*Struthio camelus*).

Although a particular mating system may appear to predominate, not all the members of a species will follow the same strategy (Krebs and Davies, 1978). The theoretical work of Trivers (1972) suggested that male and female interests would conflict in a monogamous species due to anisogamy. Females invest heavily in each gamete and their reproductive potential is largely limited by this investment. Males, on the other hand, produce sperm relatively cheaply and could potentially fertilize many more eggs than their mates can produce. Thus selection will favour males which try to fertilize any available female as long as this does not involve a reduction in the number of successful fledglings sired. Males are expected to attempt extra-pair copulations (EPCs) with the mates of other males, and if solitary females are capable of raising some nestlings to fledging, mating polygynously may also lead to a net increase in reproductive success for the male.

Male Pied Flycatchers (*Ficedula hypoleuca*) increase their reproductive output by adopting a polyterritorial strategy (Alatalo *et al.*, 1981). Having acquired a mate and fertilized the eggs, males attempt to establish a new territory some distance from the first. If a female is attracted to the new nest site, the male will fertilize the brood before

returning to the primary female. No paternal care is given to the second brood unless the first fails. The secondary female will raise fewer young due to the lack of aid but the male's net productivity is increased. In several species which are regularly polygynous, females actually benefit from choosing already mated males if their territory provides greater resources than can be obtained from unmated males (e.g. Orians, 1980). However, the principal cause of polygyny in species which are usually monogamous is a biased sex ratio which forces females to mate with paired males in order to breed at all.

The flexibility of reproductive strategies is highlighted by the Dunnock (*Prunella modularis*) in which the mating system varies temporally and spatially depending on food availability (Davies and Lundberg, 1984). Female home range size varies, being smallest when food is plentiful allowing males holding rich territories access to several females, whilst food scarcity results in an enlargement of home range size to cover the territories of several males with which the female will mate in order to gain aid in raising the nestlings. Territorial males often tolerate subordinates which help with territorial defence. These males usually manage to gain some copulations with the resident female and subsequently provide parental care in proportion to the number of matings they achieved (Burke *et al.*, 1989).

The increasing number of observational studies of colour-ringed populations has revealed previously unsuspected intricacies of bird mating systems such as the common occurrence of related helpers at the nests of Long-tailed Tits (*Aegithalos caudatus*) (Glen and Perrins, 1988). One of the most significant revelations is the extent of EPC amongst species previously thought to be monogamous. Indeed, it is best to use monogamy purely in the social context, e.g. a pair of birds which cooperate to raise their "progeny" rather than in relation to reproductive exclusivity. EPCs and intraspecific nest parasitism (see McKinney *et al.*, 1984; Yom-Tov, 1980; Rohwer, 1989) are sufficiently widespread that doubt must be cast on all estimates of lifetime

reproductive success that do not take into account the extent of these alternative reproductive strategies.

The first demonstration of the reproductive benefits which can be accrued from extra-pair copulation in birds was Burns *et al.* (1980) study of captive Mallards (*Anas platyrhynchos*). They used wild type ducks (homozygous dominant) and the recessive dusky form to form four pairs with dusky females. EPCs by males of the other morph would result in incompatible plumage types amongst the hatchlings. Several forced extra-pair copulations were observed and these resulted in 8% of the 156 progeny differing from the expected phenotype. Plumage polymorphisms are rare in natural populations though they have been used to demonstrate fostering, egg dumping and EPC in the Lesser Snow Goose (*Anser caerulescens caerulescens*) (Lank *et al.*, 1989).

Other types of genetic marker are required to examine a wider variety of species. Karyotyping has successfully demonstrated multiple inseminations of wild caught female *Drosophila pseudoobscura* (Anderson, 1974), but avian karyotypes are insufficiently variable to be of use in the analysis of parentage (Shields, 1987). Three techniques are suitable for detecting non-parentage rates in wild birds. The first which can only detect cases of non-paternity involves comparing the similarity of some morphological measurement (usually tarsus length) of nestlings with the male and female attendants. The slope of the relationship (i.e. the heritability) between young birds and their male attendants is lower than with the females, the difference in the slopes being proportional to the number of incorrectly assigned males. This method has given estimates for the extra-pair fertilization rate of 24% in the Pied Flycatcher (Alatalo *et al.*, 1984), 26% in the Swallow (*Hirundo rustica*) (Møller, 1987c) and 40% in the Indigo Bunting (*Passerina cyanea*) (Payne and Payne, 1989). It gives an estimate of the rate across the whole population but cannot identify specifically the mismatched nestlings. Starch gel electrophoresis can conclusively exclude some individuals from parentage. However, its success requires moderate levels of genetic

variation at the scorable loci, a requirement which is not fulfilled in species such as the Acorn Woodpecker, (*Melanerpes formicivorus*) (Mumme, 1985). Westneat's study of Indigo Buntings combines genetic data with extensive observations of the same population (Westneat, 1987). He found that extra-pair copulations were common (3.3%) and resulted in many fertilizations (27-42%). In addition he was able to demonstrate that neighbouring territorial males were the most likely fathers (see also Alatalo *et al.*, 1984) on the basis of similarity to the nestlings phenotype. Clearly when a males' paternity of a brood is threatened to this extent, selection will favour tactics which limit the amount of cuckoldry. Males of many species guard their mates by following them closely when possible. Even so forced extra-pair copulations have been recorded in species in which mate guarding is highly developed such as the Bank Swallow (*Riparia riparia*) (Beecher and Beecher, 1979) whilst in other species the female may escape from her partner in order to mate with another male (e.g. Burke, 1989).

An alternative adopted by males when they are forced to leave their mates unattended for ecological reasons is to mate repeatedly in an attempt to dilute or displace inseminations from other males. Both mate guarding and frequent copulations are timed to coincide with the females fertile period, which suggests that sperm competition has moulded these aspects of avian behaviour (see Birkhead, 1987, for review).

The most effective method of assaying the outcome of sperm competition is examination of the DNA sequence. Conventional RFLP analysis uses radio-labelled probes which hybridize to regions of the genomic DNA containing polymorphic restriction enzyme recognition sites. However, only two states are possible, i.e. presence or absence of the site, therefore probes must cover more than one polymorphic site if more than two marker alleles are required. Reliable parentage analysis is thus expensive and time consuming, requiring many probes and digests (e.g. Quinn *et al.*, 1987).

DNA fingerprinting provides a cheaper, faster and more effective alternative which can be applied to virtually all species using readily available human derived probes. The successful demonstration of DNA fingerprints in birds (Burke and Bruford, 1987; Wetton *et al.*, 1987) and their effectiveness in detecting cases of non-paternity has led to the application of the technique to several long-term study populations, e.g. Dunnocks *Prunella modularis* (Burke *et al.*, 1989), Purple Martins *Progne progne* (Morton *et al.*, 1990), Indigo Buntings *Passerina cyanea* (Westneat, 1990) and House Sparrows *Passer domesticus* (this study).

The House Sparrow

The European House Sparrow *Passer domesticus* is one of the most successful and widespread terrestrial birds. It is endemic to Europe, Central Asia, the Indian subcontinent and North Africa. Following introductions by man it now occurs in North and South America, Australasia, South and East Africa and many inhabited islands (Summers-Smith, 1963; Vaurie, 1956). At present it is still spreading rapidly through South America and has recently crossed the equator (Smith, 1980).

Throughout its distribution the Sparrow is an obligate commensal of man, this association probably originating during the development of early agricultural societies in the Near East (Johnston and Klitz, 1977) or the Nile Valley (Summers-Smith, 1963). The stored grain associated with early human habitations would have provided easily accessible food for the granivorous sparrows during periods of food restriction in more natural habitats. The Sparrow extended its range across Europe and Asia following the spread of cereal culture across the continent.

The precise taxonomic status of the genus *Passer* is still a matter for debate but on the basis of anatomical, behavioural and serological data it has been placed in the

family Passeridae (Old World sparrows) which form a link between the Fringillidae (true finches) and Ploceidae (weaverbirds) (Summers-Smith, 1984). The New World sparrows (Embrizidae) are more distantly related.

Since it is so common and considered a pest of some economic importance it has attracted much attention and is probably the best studied wild bird (see Summers-Smith, 1963 and 1988; Pinowski and Kendeigh, 1977; Parkin, 1987 for reviews). Many of these studies have looked into its reproductive ecology.

Juvenile males start prospecting for nest sites in their first autumn. Many of these will still be occupied by the pair that bred there during the summer as birds tend to remain attached to the same nest and will use it as a roost during the winter (Summers-Smith, 1958). Pair formation usually follows the loss of a mate, subsequently most pairs remain together for life, partly as a consequence of nest site fidelity. They are socially monogamous although occasional cases of polygamy have been observed (Summers-Smith, 1963; personal observation). Extra-pair copulations and egg dumping occur quite regularly (Cheke, 1969; D. Harper, pers. com.). Helping behaviour by adults and juveniles has been recorded in one population (Sappington, 1975). These birds were apparently unrelated to the parents and did not contribute to nesting success. Other studies have found little evidence for helping which seems to be restricted to certain geographic locations (Summers-Smith, 1963; North, 1968; Burke, 1984; personal observation).

Nestbuilding usually commences about a week before the first clutch is started. The onset of egg laying is negatively correlated with latitude (Dyer *et al.*, 1977), the proximate cues being temperature (Seel, 1968a) and photoperiod (Will, 1970). In Britain the first eggs are usually laid between April and early May. The modal clutch size is 4 eggs which are laid on consecutive days. The modal incubation period is 12 days and is carried out by the female. The male does not develop a brood patch and

merely covers the eggs to reduce cooling during the female's brief feeding bouts (Seel, 1968b; Summers-Smith, 1963).

All the eggs in a clutch usually hatch within 24 hours following 11 days incubation which does not begin until the penultimate egg has been laid. The brood is fed by both parents. Although some offspring have been raised to fledging by single parents, the efforts of both parents are usually required, hence polygamous broods are less successful. The contribution of individual parents varies greatly but females generally provide most food. The modal nestling period is 18 days and the parents, especially the male, continue to feed the fledglings for a few days after leaving the nest. The necessity of parental care during the post-fledging period may be why the modal clutch size is lower than that which gives the highest fledging success (Schifferli, 1978) otherwise it might be accompanied by higher post-fledging mortality. Some evidence for the production of earlier and larger clutches by older females has been gathered (Seel, 1968a). The subsequent clutches follow soon after the previous brood has fledged and may even overlap it (Lowther, 1979). Three broods may be raised before nesting activity starts to decline in the latter part of July. The last clutches are produced in early August (Summers-Smith, 1963).

Approximately 50% of nestlings survive to fledge, the majority of losses occurring through starvation which has a density dependent effect in large broods. Juvenile mortality is also high, 80% of fledglings fail to breed, most starve or fall victim to predators during the first few weeks after leaving the nest (Summers-Smith, 1963; Seel, 1970). Unusually for a passerine, most adult mortality occurs during the breeding season, although a similar pattern is found for the Starling *Sturnus vulgaris* and Blackbird *Turdus merula* (Lack, 1968, p. 300). The Sparrows use of food provided intentionally or otherwise by man lessens the importance of overwinter mortality. However extreme climatic conditions can result in heavy losses.

The classic study by Bumpus (1899) of mortality among a flock of North American Sparrows during a severe winter snowstorm led him to hypothesize that they had been subject to stabilizing selection for body size. The data have been subsequently reanalysed by Grant (1972) and Johnston *et al.* (1972) who concluded that females had indeed been subject to stabilizing selection but the males had been directionally selected for larger body size.

Morphological variation in natural and introduced populations is significantly correlated with latitude. Introduced populations in North America comply with Allen's, Bergmann's and Gloger's ecogeographic rules although European birds are smaller in more northerly populations, but agree with Allen's and Gloger's rules. More continental populations, however, comply with Bergmann's also. Sexual dimorphism increases with latitude (Johnston and Selander, 1973) but diminishes above 45°N in Europe as body size also declines. Downhower (1976) has suggested that small body size allows females to utilize currently available food more efficiently rather than having to delay breeding whilst body weight is regained. This will be more important in places where the breeding season may be short or early breeders are favoured. Burke (1984) has found assortative mating for body size, specifically for tail length and body weight at one of his study sites, but there was no departure from random mating with respect to the biochemical polymorphisms investigated.

Biochemical polymorphisms have been used by several workers to investigate genetic differentiation between House Sparrow populations. The earliest by Klitz (1972) used an array of loci which are relatively invariant and led him to suggest that introduced populations in America tended to be monomorphic, a surprising result considering the rapid morphological evolution of American populations. Subsequent studies by Manwell and Baker (1975) and Cole and Parkin (1981) have demonstrated a higher degree of variability which has allowed the divergence between populations to

be compared with the predictions of models based on gene flow and random genetic drift.

Endemic populations from the East Midlands which are effectively continuous across the whole area fit a model of isolation by distance (Parkin and Cole, 1984) whereas populations restricted to the area around individual farmsteads in Kansas agree with the predictions from a “stepping stone” model of population structuring (Fleischer, 1983). Birds from Australia and New Zealand show a degree of differentiation consistent with population bottlenecks following introduction leading to the loss of some rare alleles found in European populations (Parkin and Cole, 1985). Norwegian sparrow populations are more differentiated than other European populations as they are isolated in towns separated by areas which are very inhospitable. Some populations are small and this has resulted in inbreeding and greatly increased random effects (Bjordan, 1986). Because dispersal is generally of the order of 1 - 2km, gene flow is restricted (Summers-Smith, 1963; Fleischer, 1983).

Aims of the Study

The extensive literature on all aspects of the population biology of the House Sparrow makes it an ideal species for an intra-population investigation of genetic variability. Heritable variation has been detected in morphology and at biochemical loci (reviewed in Parkin, 1987) and in minisatellite DNA sequences (this study). The following chapters will expand on Burke's (1984) study of the Brackenhurst colony by attempting to identify the causes and consequences of variation and applying the information gained to an investigation of the reproductive biology of the population.

The aims of the study are:-

- 1) The monitoring of reproductive success and the collection of blood samples and metric data from complete family groups of House Sparrows (Chapter 2).
- 2) To confirm the inheritance patterns of the electrophoretic loci studied by Burke (1984) and to determine the cause of the apparent segregation distortion at two loci (Chapter 3).
- 3) To estimate the amount of extra-pair paternity using the data collected from incompatibilities between nestlings and attendants (Chapter 3).
- 4) To develop DNA fingerprinting as a tool for paternity exclusion in the House Sparrow (Chapter 4).
- 5) To use fingerprinting to identify factors which influence the success of cuckoldry attempts (Chapter 4).
- 6) To identify the causes and consequences of metric variation in four traits: tarsus length, weight, wing and tail lengths, and if feasible to estimate the heritabilities of these traits (Chapter 5).

CHAPTER 2

THE BRACKENHURST POPULATION

The Study Site

The Nottinghamshire County Council Agricultural College at Brackenhurst, near Southwell, lies in an area of undulating farmland 18km north east of central Nottingham (Fig. 2.1). The College provides training in most aspects of farming practice, maintaining an appropriately wide variety of crops and livestock; the principal crops being winter and spring sown cereals, with large areas of green pasture for the dairy herd which are the most numerous livestock. Several sheep, pigs and chickens were also present at various periods.

The nestbox population is centred on Home Farm, a cluster of milking parlours, cattle pens and storage facilities associated with the dairy herd. These buildings form one group of nestbox sites, the other 60m away is the Workshop Department where the farm vehicles and machinery are serviced and stored (Fig. 2.2a and b). The two areas, BH and BG respectively, are separated by houses belonging to the farm staff. A small proportion of the Home Farm colony built inaccessible nests in the eaves of these houses. Other houses, student accommodation and farm buildings which also provided potential nesting sites, were present within 500m. The town of Southwell (population = 5,000) lying 1km north of Home Farm, is likely to be a major source of recruits to the population as farmland acts as a sink for the excess production of suburban house sparrows.

Nestboxes were first erected at Brackenhurst by Burke in 1980. The number of boxes available in each year, with the number of nesting attempts and breeding adults, is shown in Table 2.1. Boxes were built to a common design to minimise variation in nest site quality (see Fig. 2.3). The location of nestboxes was determined by several requirements:- All boxes had to be easily accessible with an 18' extending aluminium

Figure 2.1

Extract from an Ordnance Survey Map containing the Brackenhurst study site (BR). Spot heights and contours are shown in metres.

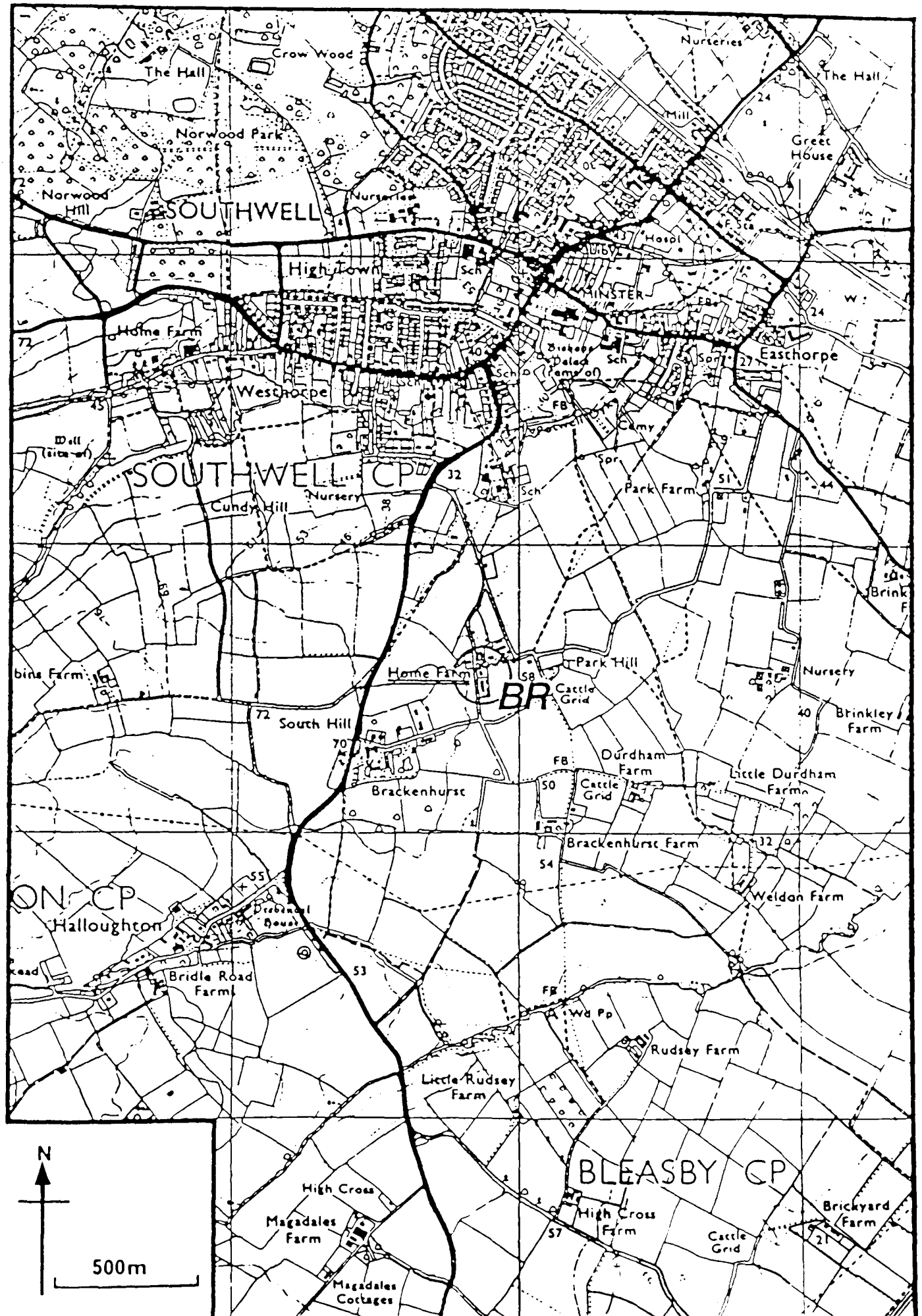


Figure 2.2a

The arrangement of nestboxes at Home Farm

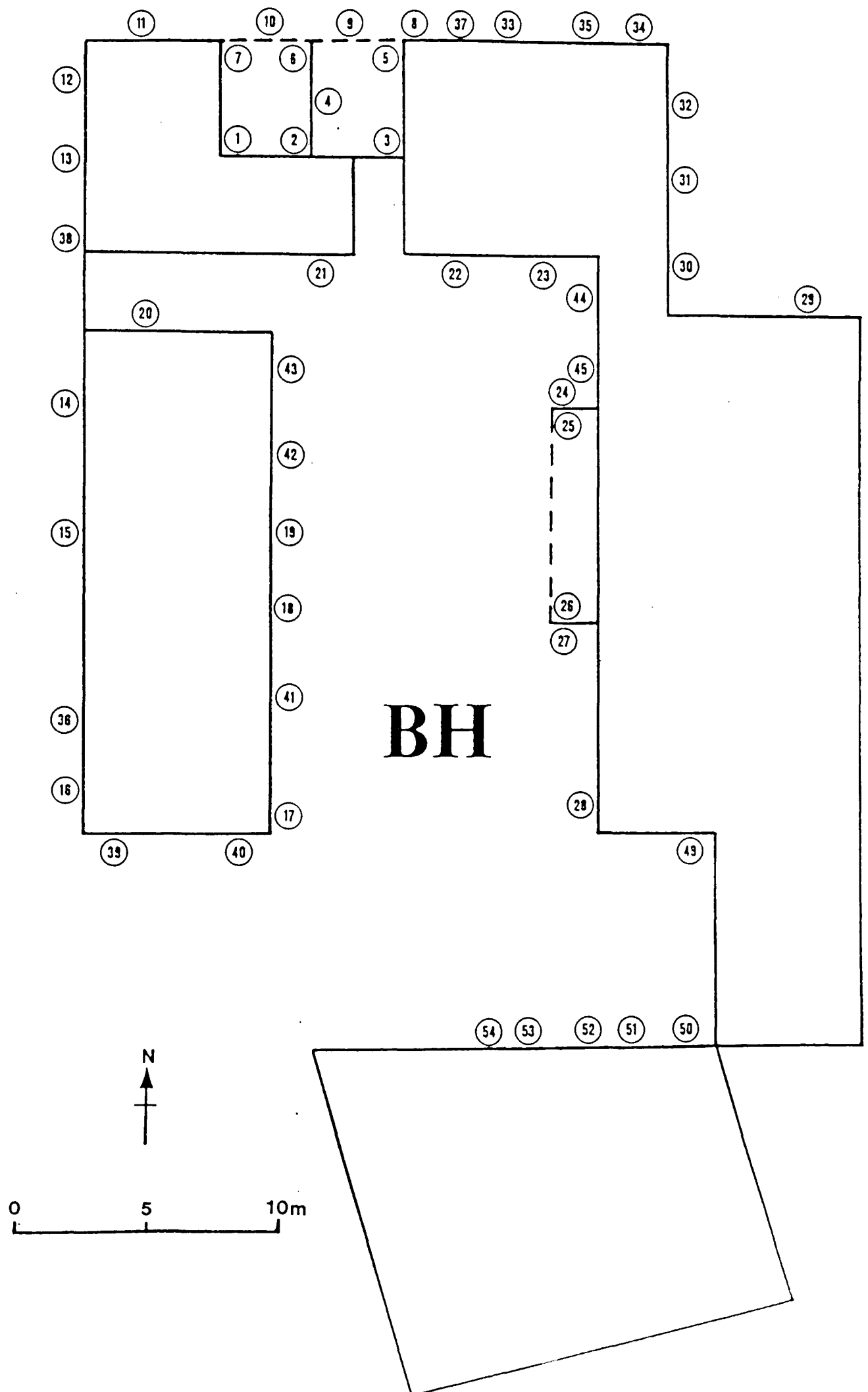


Figure 2.2b

The arrangement of nestboxes at the Brackenhurst Garages

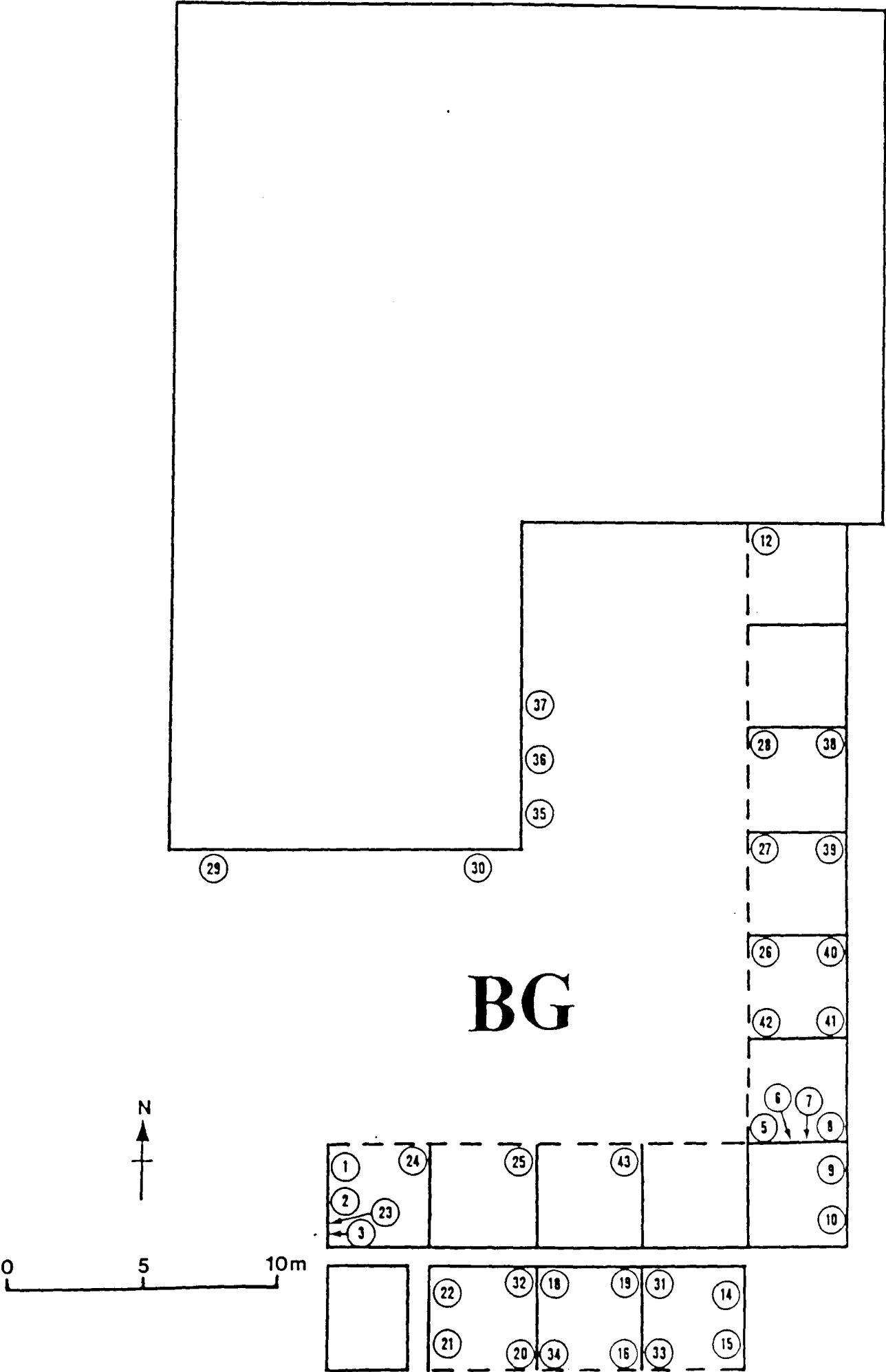


Table 2.1

The box usage, number of clutches and number of individually identifiable breeders at Brackenhurst in each year of the study

Year	Number of Clutches	Number of boxes used	Number of boxes available	% used	Number of ringed breeders	
					M	F
1980	27	15	35	42.8	5	6
1981	82	40	63	73.0	22	27
1982	104	51	63	81.0	20	36
1983	101	51	63	81.0	20	25
1984	84	42	63	66.7	17	26
1985	91	48	80	60.0	25	29
1986	107	59	80	73.8	44	42
1987	79	44	90	48.9	38	39
1988	37	26	90	26.3	13	12

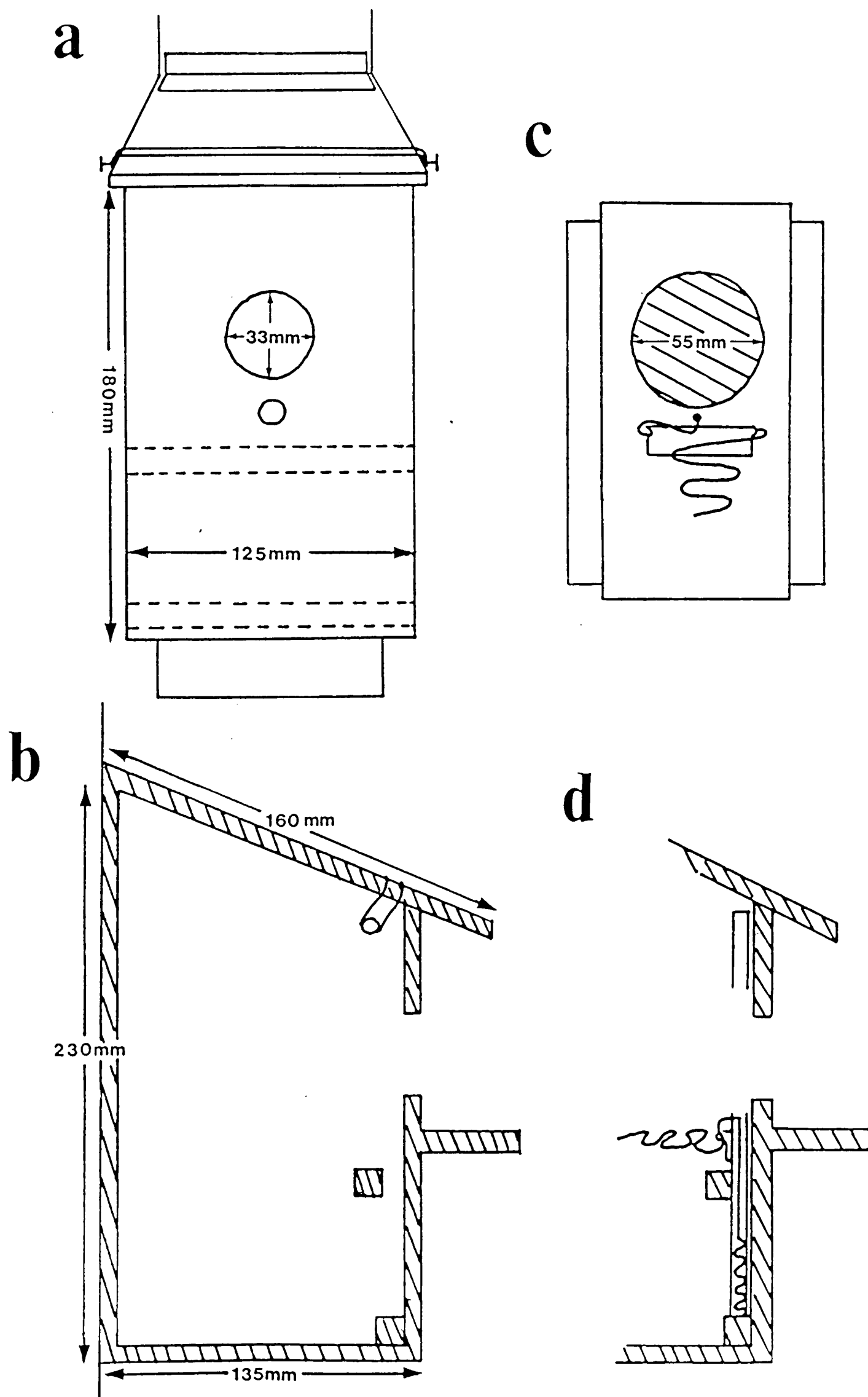
Figure 2.3

Nestboxes were constructed from 6mm exterior grade plywood. The component panels were joined with fine panel pins and a waterproof wood adhesive. 15 x 15mm ramin beading provided extra strength along the front bottom join and provided a support for traps (see (d)). The box lids were hinged with stapled strips of 40mm wide elasticated seat webbing. The lid was held closed by an elastic band looped between nails on each side of the box. A 40mm length dowel (6mm in diameter) was attached below the entrance to provide a perching site on which the birds colour rings could be read during feeding visits. The external dimensions of the box are shown.

(c) shows a rear view of a spring loaded trap used to capture adults during the nestling period. The trap was painted black, inserted into the slot behind the entrance (d). The spring loaded panel was depressed below the level of the entrance and locked in position by the hinged treadle which was then camouflaged with nest material. On entry a bird will step on the trigger mechanism releasing the sliding panel.

(see over)

Figure 2.3



ladder and orientated such that birds visiting the box could be seen from a parked vehicle with 10 x 50 binoculars. Whenever possible, the boxes were arranged so that several could be observed simultaneously.

Boxes were added throughout the study to areas which were favoured by sparrows nesting in natural sites. Persistent nest removal and the blocking of natural sites was carried out before April when the first eggs were laid in an attempt to encourage the birds to use nestboxes. Boxes placed less than 3' above horizontal surfaces were not used, but virtually all other boxes were occupied at some time during the study. Orientation seemed to have little effect on box usage. The two sub-areas differed in availability of potential nestbox sites. Boxes in area BG were attached to concrete roof supports inside asbestos panelled tractor shelters, whilst all but seven boxes in the BH area were attached to exterior walls with masonry nails.

Monitoring of Nestboxes

Each year, usually in February, the nestboxes were cleared of old nesting material, which would otherwise accumulate and harbour overwintering nest parasites, repaired and creosoted. All boxes were checked at regular intervals commencing in late March, initially at 4-day intervals until the first egg was found (8 - 28 April). Subsequently, each box was examined 3 times a week. Changes in the amount of nest material were recorded to aid prediction of the onset of laying. If eggs were present their number and whether they were being incubated was noted. On the first visit after hatching a single claw was trimmed with a pair of iris scissors to allow subsequent identification. Every nestling was weighed on each visit to assess growth rate and fitness. Visits to nestboxes were carried out as quickly as possible to minimize disturbance. If adults were found inside the nestbox the contents were left unchecked.

Generally, though, adults would leave the box as the fieldworker approached and would re-enter shortly afterwards.

The data from box checks were analysed for evidence of temporal or spatial differences in reproductive success. Clutch size, hatching success and nestling survival were examined using an analysis of variance of the years from 1985 to 1988 (Table 2.2). Clutch size and hatching success were constant throughout, but nestling survival (determined as the proportion of hatchlings surviving to fledge) increased steadily throughout the study ($F_{3,273} = 4.90$, $P < 0.005$). The basis of this trend is presumably an improvement in the food supply because virtually all nestling mortality at Brackenhurst was through starvation. The feeding rates of nest attendants appeared lower during periods of high nestling mortality following periods of extreme weather, cold, rain or drought and in 1985 a rash of nest failures occurred soon after the surrounding fields were sprayed with insecticide.

Access to food might result in spatial variation in nesting success. Dawson (1972) detected a decrease in number of fledglings/nest/annum at distances over 100m from farmyards. Interestingly, adults nesting in the BG area were frequently observed foraging in the feeding troughs at BH whilst birds from the BH boxes were very rarely seen near BG. The consequences of nestbox location and food accessibility were examined as above for years, but no significant differences were found other than a slightly higher fledging success at BH in 1986 (Table 2.3). Foraging sites for nestling food were found all around both areas.

Trapping

Since the Brackenhurst nestbox colony was established in 1980, 1,226 nestlings and 1,024 free flying birds have been handled and ringed. Independent birds

Table 2.2

Between year analysis of variance of the mean brood statistics (\pm s.e.m.) for clutches incubated for the normal period

Year	N	Eggs	Hatch	Proportion Hatching	Fledge	Proportion Fledging
1985	78	4.256	3.692	0.863	1.705 \pm 0.183	0.456 \pm 0.047
1986	100	4.170	3.830	0.922	1.930 \pm 0.146	0.523 \pm 0.040
1987	70	4.214	3.786	0.903	2.443 \pm 0.171	0.651 \pm 0.043
1988	28	4.107	3.643	0.887	2.464 \pm 0.238	0.710 \pm 0.061
85-88	276	4.199 \pm 0.049	3.761 \pm 0.061	0.897 \pm 0.011	2.051 \pm 0.091	0.555 \pm 0.024
F-ratio		0.30	0.42	1.77	4.00	4.90
P		N.S.	N.S.	N.S.	<0.01	<0.005

Table 2.3

ANOVA of brood data from the two sub-areas of Brackenhurst

Year	Number of Broods		d.f.	F				
	H	G		Eggs	Hatch	Fledge	Proportion Hatching	Proportion Fledging
1985	53	25	1, 76	0.90	3.25	0.15	2.51	0.01
1986	70	30	1, 98	0.06	1.46	4.61*	1.87	2.87
1987	59	11	1, 68	0.08	0.69	0.24	0.63	0.01
1988	20	8	1, 26	0.01	0.82	0.57	0.97	2.23

* = $P < 0.05$

were caught using nets or nestbox traps. The relative importance of each technique varied between years, with a greater emphasis on mist netting in the latter years (Table 2.4a).

Mist nets were used to catch a random sample of birds to gain demographic and allele frequency data. During August, mist nets were set in the fields surrounding Home Farm where large flocks accumulated to feed on the ripening grain. These flocks consisted mainly of dispersing juveniles and probably included a number of adults from other colonies. Following the harvest, the flocks dispersed as birds returned to their nesting sites. From July to May nets were used across the openings to cattle pens in which large numbers of birds regularly fed. The majority of birds caught during this period lived in the immediate vicinity of the Home Farm study site. Bat nets were also used at this time to catch birds feeding in individual pens. The net consisted of a bamboo framework 3' x 5' supporting 5' x 5' piece of mist net; this would be held across the open upper half of stable doors. Mist nets were used following the advice in the BTO ringers manual (Spencer, 1976).

Netting was rarely carried out during the breeding season, to reduce the risk of delaying the return to nest of breeding adults during incubation or the early nestling phase. In some areas mist nets were used to catch particular adults visiting nestboxes. The large open fronted tractor sheds in which many of the BG boxes were situated were ideal for the purpose. In these cases nets were erected for short periods not exceeding 30 minutes to prevent disruption to other breeding pairs.

Unringed adults which succeeded in raising nestlings were caught using spring loaded nestbox traps adapted from a design by C. Perrins. Due to the risk of desertion associated with catching adults within nestboxes, trapping was delayed until the nestlings had been ringed and bled about four days before fledging. At this age desertion is very unlikely and would not greatly alter the fledging success.

Table 2.4a

The number of previously unringed individuals captured by netting and nest trapping each year

Year	Trapping	Netting
1980 - 84	92	317
1985	19	203
1986	23	139
1987	9	111
1988	2	109
1985 - 88	53	562

Table 2.4b

The number of different individuals in each age class caught with nets and traps

Each individual is included in only one age group in each year but may have been both trapped and netted in the same year in which case it appears in both categories and several appear in the tallies for more than one year.

Year	Nest Trap		Mist Netting			
	Adult Male	Adult Female	Juvenile	Unknown Age	Adult Male	Adult Female
1985	12	24	13	26	123	91
1986	21	31	52	7	103	59
1987	6	13	39	34	73	61
1988	1	4	51	11	83	37

The trap was fitted into a slot behind the front wall of the nestbox into a space kept clear of nest material by a plywood filler when the trap was not in use (see Fig. 2.3). When the nestlings reached the appropriate age, they were removed from the box, the spacer and trap were exchanged and the trap set with the trigger mechanism camouflaged with nest material. The box was watched from a distance so that a bird could be retrieved from the box as soon as it had entered and activated the trap. A large piece of cloth would then be placed over the nestbox and the lid partially opened so that the cloth blocked off both light and possible escape for the bird whilst allowing a hand to be inserted into the box. If the mate of the intended bird was caught the trap was reset and the bird held in a cloth bag until either the other adult was caught or 30 minutes had elapsed. At the completion of a trapping attempt, the trap was removed and the nestlings returned. At least 48 hours were allowed between successive trapping attempts at any one box.

Prior to 1985 the population was largely composed of unringed birds which prevented the identification of the attendant adults until the latter stages of the breeding cycle when trapping could be attempted (Table 2.5a and b). This resulted in several erroneous assignments of parentage (T. Burke, pers. comm.). On two occasions after 1984 birds other than the attendants were trapped in nestboxes. Whenever possible observation of feeding visits by ringed adults was used to assign attendancy with greater reliability. None of the families analysed between 1985 and 1988 by DNA fingerprinting were likely to have involved the mis-identification of the attendant adults. In addition, the high proportion of pairs ringed during the fertile period enabled the collection of data on copulation behaviour of birds of known breeding status (see Chapter 4).

The use of nestbox traps was restricted to those cases where the adult could not be caught using alternative methods. The removal of the nestlings from the box was

Table 2.5a

The parentage of successful breeding attempts where the attendants were individually identifiable before incubation and before fledging

a)

Year	No of broods	Percentage of males ringed before attempt		Percentage of females ringed before attempt		Both attendants ringed %
		Initiated	Ended	Initiated	Ended	
80 - 84	245	22.0	41.6	30.6	55.5	33.1
1985	47	53.2	70.2	55.3	87.2	61.7
1986	74	71.6	85.1	66.2	87.8	75.7
1987	58	91.4	94.8	87.9	100.0	94.8
1988	26	73.1	76.9	80.8	84.6	73.1
85 - 88	205	73.2	83.4	71.7	90.7	77.6

Table 2.5b

The number of fledglings produced in each year for which the attendant adults were identified

b)

Year	Number of nestlings	Attendant Identified			
		Neither	Male only	Female only	Both
80 - 84	642	216	68	149	209
1985	133	3	9	32	89
1986	206	18	16	16	156
1987	175	0	2	9	164
1988	70	10	2	8	50
85 - 88	584	31	29	65	459
80 - 88	1226	247	97	214	668

necessary as they would otherwise have triggered the trap mechanism. Adults usually feed nestlings older than 10 days from the nestbox perch through the opening and many were reluctant to enter the unoccupied box. A greater proportion of females were caught using this method, females make slightly more feeding visits than males at this stage whilst the males encourage the nestlings to fledge by calling from outside of the box (see Table 2.4b).

Rather more surprising was the bias towards males in the mist netted sample. Considering only birds in adult plumage known to have survived one winter, there is a clear excess of males. The deviation from a 1:1 sex ratio is significant in 3 out of 4 years (see Table 2.6). This may be a true reflection of the composition of the population or might reflect the greater ease with which males are netted, possibly because they spend more time feeding in the netting areas.

If males are more common at Brackenhurst this could result from a biased sex ratio at birth, differential mortality before the attainment of adult plumage or a shorter life expectation for adult females. In 1988 the very strongly biased sex ratio is almost certainly partly due to a poisoning incident. During the winter of 1987-1988 some grain treated with Panogen M, a mercurial fungicide, was left exposed in the BG area for several months by the farm staff. This became a popular food source for the colony as evidenced by the accumulation of sparrow faeces around the grain spill. During the peak of laying activity in late April many females disappeared from the population, several leaving incomplete clutches. The mobilization of food reserves and increased feeding rate at this time will have increased the concentration of the toxin in the circulation resulting in death. This incident greatly reduced the number of pairs and resulted in an influx of unmarked birds, which might represent a floating population of previously unmated yearlings.

Table 2.6**Variation in sex-ratio of mist-netted samples**

The number of adult males and females netted each year was examined using a G test for goodness of fit to a 1:1 ratio. The apparent change in sex ratio between consecutive years was tested with χ^2

Year	Number of males	Number of females	G _{1:1}	Probability	Heterogeneity between years χ^2 (df)	
1985	123	91	4.79	P < 0.05	1.432	(1)
1986	103	59	12.07	P < 0.001	2.521	(1)
1987	73	61	1.07	P < 0.5	5.764 *	(1)
1988	83	37	18.02	P << 0.001		
1985-88	285	206	12.75	P < 0.001	7.271	(3)

* = P < 0.05

Twenty males and eleven females ringed as nestlings at Brackenhurst subsequently bred in the nestboxes. Of these 40% of males and 22% of females did not attempt to breed until their second year. In general, once a nest site has been acquired it is used in subsequent years. Failing this, the distance to the new nesting site is usually very small, e.g. the adjacent box. The limited breeding dispersal ensures that most birds surviving from one season to the next will continue to use the boxes and be identified in due course. Thus, it is likely that a proportion of yearlings delay their first breeding attempt until their second year. However, the possibility that they used natural nest sites cannot be excluded. In 1988 three adult males were known to have remained at the nestbox which they had used in the previous year but failed to attract a new female following the disappearance of their mate. Therefore, a proportion of full adult males also remained unmated in 1988.

Biased sex ratios existed before the poisoning incident as well. Possible causes were examined using the recovery data of ringed individuals. Forty-two nestlings ringed at Brackenhurst between 1984 and 1987 were recaptured after their first winter. The mean time between fledging and last known sighting of these individuals did not vary between the sexes (mean 531.5 ± 52.4 days, $N = 26$ males, 513.8 ± 94.1 days, $N = 16$ females, $F_{1,40} = 0.03$, N.S.). The absence of longevity differences in yearlings implies that the bias must arise earlier in the lifecycle. Female dispersal distances are usually greater than males which could account for the slightly lower number of females recovered as yearlings. However, immigration by others would normally rectify the discrepancy. Once recruitment occurs, there is no difference in the period between the first and last sightings of adults (249.9 ± 20.9 day, $N = 255$ males, 217.2 ± 19.9 days, $N = 201$ females). Therefore, a solution to the sex ratio bias requires sexing of nestlings to see if differential mortality occurs before the yearling plumage is acquired.

Ringling

On first handling all free flying birds were measured (see Chapter 5) and ringed with a numbered BTO ring and a combination of coloured celluloid rings. The plastic rings supplied by A.C. Hughes were of eight colours; white, mauve, red, black, blue, yellow, orange, green and pink. Three colours, mauve, black and pink were difficult to identify under field conditions with a telescope and so were restricted to birds ringed as juveniles in grain field flocks, relatively few of which were recaptured. Under poor light conditions, blue and green could also be confused and so were used infrequently. Mistaken identification of birds due to misreading of colour rings was rare and not associated with particular colour combinations. Colour rings were sealed with acetone in an attempt to prevent ring loss. However, some birds succeeded in removing some or all of their colour rings within months. Three colour rings were fitted to each bird so that it carried two rings on each leg. This allows for 2048 combinations with 500 being usable on adults of each sex. Combinations using less than three colour rings were not used due to possible confusion with other birds that had lost rings.

Blood Sample Collection

Blood samples were collected after ringing and measurement. The bird was held in the left hand with the neck between the 1st and 2nd fingers, tarsi between the 3rd and 4th and the wing extended by the 2nd and 3rd. Ethanol was applied to the feathers above the right jugular vein. The dampened feathers could then be smoothed apart leaving the vein exposed between two feather tracts which run longitudinally down the neck. A further application of ethanol to the skin over the vein both disinfected the area and increased the blood flow. Slight pressure could then be applied to the vein in the region of the crop. The vein would dilate sufficiently to allow insertion of a 25g 16mm hypodermic needle fitted to a preheparinized 2ml syringe at a

shallow angle with the base of the hypodermic facing into the flow of blood. The plunger of the syringe was then slowly withdrawn until 0.4ml of blood had been collected. The left thumb was then placed over the vein at the point of entry and the hypodermic carefully withdrawn. After 20 seconds a tissue paper swab would be applied if there was evidence of bleeding. The swab was held in place until all loss ceased. The bird was again examined after a further minute. Adults were then released and nestlings placed in a cotton bag until their brood mates had been processed.

The hypodermic was removed from the syringe and the blood transferred to a 1.4ml eppendorf tube labelled with the BTO ring number. The tube was gently agitated to ensure thorough mixing of blood and heparin before being placed in a cold box at 4°C with ice packs for transfer back to the lab. The subsequent treatment of the blood sample prior to electrophoresis and fingerprint analysis is given in Chapters 3 and 4 respectively.

CHAPTER 3

THE PROTEIN POLYMORPHISMS

Population geneticists have in the main used electrophoretically detectable protein polymorphisms to quantify genetic variability. Amongst others, Wright and Nei, have developed statistics which describe genetic heterogeneity within and between populations (Nei, 1975). These have shown that most variation occurs within populations. Even so, there is insufficient to characterize individuals using protein electrophoresis alone. The paucity of variation has limited the power of starch gel electrophoresis in population analyses that require individual specific arrays of genetic markers, e.g. the assignment of parentage and estimation of coefficients of relatedness.

However, long term population studies which involve sampling several generations can reveal a great deal about the processes involved in the maintenance of polymorphisms and the spread of alleles. They are greatly strengthened when the genealogy and reproductive history of individuals is known, this in turn allows the confirmation of Mendelian inheritance of phenotypes scored from gels. The sampling of entire families also permits more stringent analyses of linkage and early acting selection than can be attempted with random population samples or incomplete family data sets (Redfield, 1973).

Electrophoretic surveys of complete families can also demonstrate the genetic consequences of alternative reproductive strategies. Amongst birds the commonest social system is the monogamous pair because the successful rearing of nestlings to independence usually requires a considerable investment in parental care by both adults. However, as Trivers emphasised, the sexes differ in their investment in gametes. For males this is relatively small and their reproductive success is limited primarily by their ability to acquire mates. Since selection favours those individuals that behave so as to maximize their reproductive success, males should attempt to mate with any fertile female including the mates of other males (Trivers, 1972). Females, however, are

limited by their ability to produce eggs and so the variance in female reproductive success and hence their contribution to future generations may be lower. Thus the identification of all individuals affected by alternative reproductive strategies is necessary if a precise genetic analysis of a population is contemplated.

Frequent extra-pair matings or brood parasitism may alter the effective population size if individuals which have apparently bred successfully are raising the offspring of other birds. More importantly, undetected cases of cuckoldry and brood parasitism which are included in the analysis of inheritance patterns can produce anomalous results.

Burke's survey of the Brackenhurst and Sutton Bonington House Sparrow populations involved seven polymorphic loci resolvable from blood which are described in more detail later. He found that segregation at four loci, 6-Phosphogluconate dehydrogenase (6PGD), a dipeptidase (PEPD2), iso-citrate dehydrogenase (IDH) and a tripeptidase (PEPT) was in accordance with simple Mendelian inheritance. An esterase (EST2) possessed three codominant alleles and one or more recessive null alleles but of particular interest were the two remaining loci, another dipeptidase (PEPD3) and transferrin (GP), at which segregation distortion occurred in all sex, site and year classes. At each locus the common allele was transmitted more frequently than a rare allele by heterozygous parents.

Burke considered several possible causes ranging from laboratory error to selection. Mis-scoring of gels would have affected parent birds as frequently as their offspring resulting in a significant excess of nestlings incompatible with their parents genotype due to possession of the unscored rare parental alleles, when in fact genetically mismatching nestlings possessing these alleles occurred at the expected frequency relative to mismatches at other loci.

Examination of the nest record data showed no differences in initial clutch size or fledging success for broods raised by carriers of the affected alleles. Therefore, simple fitness differences during the nestling period were not compensated for by larger clutches. Density dependent selection could also be dismissed as distortion was apparent in broods which had suffered no mortality. Frequency dependent selection could result in mortality in broods with an initial excess of rare heterozygotes whilst leaving unaffected those where carriers of the rare alleles were in a minority.

An alternative hypothesis invokes prezygotic or gametic selection. However, in most cases where this has been implicated including the t complex in *Mus* (Bennett, 1975) and the \underline{D} factor in *Aedes aegypti*^p (Hickey and Craig, 1966) segregation distortion was confined to one sex which was not the case in Sparrows. Whatever the mode of selection, if such was acting, it was clearly unbalanced at the time of fledging and no evidence of later advantages conferred by the rare alleles was found.

In an attempt to clarify the problem of distorted segregation ratios and other questions raised by Burke's study, blood sampling was continued at both populations during 1983 and 1984 and is continuing at Brackenhurst. The concentration of effort on the Brackenhurst colony dramatically increased the proportion of complete families sampled and provided material for a detailed analysis of breeding success at the farm between 1985 and 1988.

The following sections describe the techniques involved in starch gel electrophoresis and the enzyme polymorphisms themselves. Segregation and linkage are analysed using confirmed family groups, and genetic variation between age, sex and year classes will be examined. Having determined the characteristics of the polymorphisms, they will be used to estimate the rate of cuckoldry within the Brackenhurst population.

METHODS

Sample Preparation and Storage

Whole blood collected in the field was transferred to the laboratory in a cool box at 4°C, where two 15 µl aliquots were removed for DNA extraction (see Chapter 4). The remaining blood was separated into plasma and cellular fractions by centrifugation for 20 minutes at 2000 g. The plasma was then transferred to a fresh eppendorf labelled with the appropriate BTO ring number, and the cellular pellet was resuspended by vortexing in an equal volume of 0.1% Triton X-100. Both fractions were then stored at -80°C until the end of the field season.

On completion of the year's fieldwork both fractions were thawed on ice. The resuspended cells were lysed by freezing and thawing in the presence of Triton releasing soluble proteins from the cells. The cellular debris was then pelleted for 40 minutes at 2000 g in a cooled centrifuge at 4°C leaving a clear lysate. To minimise the number of freeze-thaw cycles involved in electrophoresing the samples for each enzyme and any necessary reruns, 30 µl aliquots of plasma and lysate were transferred into individual wells of 96 sample microtitre plates. Six replicate plates were made of erythrocyte lysates and four of plasma. Samples were plated in BTO ring order and each plate was labelled with a code number, sealed with strips of masking tape over the wells and then frozen at -80°C until required for analysis.

Electrophoresis

Isozymes are separated by applying a direct electrical current across a gel into which filter paper inserts soaked in the sample have been placed. The rate at which each molecule moves through the porous gel is dependent on its net electric charge, size and shape and also on the concentration of ions in the gel and tank buffers which carry much of the current. The electrophoretic buffers, apparatus and methods used were

essentially those given in Burke (1984), which were in turn adapted from protocols in Harris and Hopkinson (1976), Shaw and Prasad (1970) and Cole and Parkin (1981). They are described briefly below.

Gel Preparation

A variety of gel and tank buffers were used, the recipes of which are given in Table 3.1. Gels were prepared by boiling a 10% (weight/volume) hydrolysed starch/gel buffer solution in a 2l flat bottomed spherical flask over two bunsen burners. The flask was swirled constantly to prevent starch from adhering to the glass. Heating continued until the solution cleared and viscosity decreased at which point the flask was attached to a water vacuum pump and degassed in a plastic tank to reduce the dangers from implosion. When the solution was boiling vigorously and all small bubbles had disappeared the vacuum was slowly released and the molten solution poured into perspex moulds with internal dimensions 185 x 100 x 6 mm lying on a 220 x 130 mm glass plate. Approximately 150 ml of solution was used to overfill each mould leaving a pronounced meniscus. A further glass plate of similar dimensions was then placed on one edge of the mould and lowered gently forcing out any air bubbles and excess starch. Gels were left to set overnight, or transferred to a cold plate for at least one hour if required for use that day.

Sample Loading

When the gel had set, the top plate was removed and a row of 32 slots were cut into the starch with the sharpened end of a 3.5 mm wide microspatula. The insert line was cut parallel to the long axis of the gel either 2 cm from the edge for anodally

Table 3.1

Buffers used in the starch gel survey

Buffer System	pH	Molarities	MW	g/l	Gel dilution	Tank dilution	Reference
Phosphate-Citrate (PC)	7	0.245 NaH ₂ PO ₄ 0.15 M Citric Acid 0.6 M NaOH	156.01 210.14 40.00	38.22 42.03 24.00	1:79	1:0	1 & 2
Phosphates (P)	7	0.187 M NaH ₂ PO ₄ 0.313 M Na ₂ HPO ₄	156.01 141.96	29.25 44.38	1:39	1:4	1 & 3
Tris-Phosphate (TP)	7.4	0.5 M Tris 0.5M NaH ₂ PO ₄	121.10 156.01	60.55 78.00	1:99	1:4	1 & 2
Gahne Solution A (G)	8.5	0.06 M LiOH 0.229 M Boric Acid	41.96 61.83	2.52 14.16	10:54 (A:B)	1:0 (A)	1 & 4
Solution B	8.5	0.079 M Tris 0.007 M Citric Acid	121.10 210.14	9.57 1.47			

1. Burke (1984)
2. Harris & Hopkinson (1976)
3. Shaw & Prasad (1970)
4. Gahne (1966)

migrating enzymes or 6.5 cm in for IDH (which migrates cathodally). Handcut Whatmann 3MM paper inserts (4 x 6 mm) were soaked briefly in the sample wells, then excess liquid was blotted onto scrap Whatmann paper prior to loading into the gel slots with fine forceps.

Sub-banding on peptidase gels was reduced and resolution improved by addition of 10 μ l of a 10 mg/ml solution of Dithiothreitol (DTT) to each 30 μ l sample, mixing, and incubation at 37°C for one hour. The remaining loci were scored from untreated samples.

Electrophoretic Apparatus

The electrophoresis rigs consisted of two perspex tanks of 625 ml capacity each containing a platinum electrode the length of a starch gel. A copper cooling plate was rested on a glass plate between the tanks onto which the loaded gel was placed. The gel surface was covered with a sheet of cling film which insulated the gel from a further cooling plate. A 12 mm wide strip of gel was left exposed by the upper plate providing a contact point for J-cloth wicks soaked in buffer which completed the circuit between the tanks and the gel.

Current was supplied by Heathkit 1P-17 regulated D.C. powerpacks. Details of runtimes and applied voltages are given in Table 3.2. Water at 4°C was circulated through the cooling plates for the duration of the run.

Table 3.2

Running conditions used for each locus in the starch gel electrophoresis survey

Protein	Buffer	Voltage (V)	Current (mA)	Duration	Migration
IDH	PC	110	75	4	Cathodal
6PGD	P	110	100	5	Anodal
PEPD2 } PEPD3 } PEPT }	TP	200	75	3.5	Anodal
GP	G	150-300	75	4	Anodal

Gel Staining

At the end of the run the former and inserts were removed and the areas of the gel which had been in contact with the wick were cut off, as was the bottom left hand corner to aid orientation of the stained gel. The gel was inverted onto a dry glass plate and two plastic strips half the thickness of the gel were aligned alongside. A piece of fine nylon fishing wire was stretched between the index fingers and drawn through the gel using the strips as guides. The bisected gel was separated and placed on staining trays with the cut face uppermost.

Transferrin was visualised using a non-specific general protein stain consisting of a filtered solution of 0.2% (W/V) Amido Black 10B and 0.4% (W/V) Nigrosine in 50:50:10 methanol:water:acetic acid. The gel slice was soaked overnight in 150 ml of stain and then rinsed with water before destaining with several changes of methanol, water and acetic acid solution. The other enzymes were stained using agar overlays as described in Table 3.3. Up to 3 hours were required for overlays to develop into scorable zymograms, examples of which are shown in the next section.

RESULTS

Description of the Polymorphisms

The nomenclature used follows that developed by Burke (1984) who named the alleles alphabetically with the fastest being A regardless of whether the isozymes migrate cathodally (i.e. IDH) or anodally. Several new rare alleles were discovered in the larger samples collected subsequent to Burke's survey. Where new alleles migrated between previously discovered ones the designation X was allocated.

6-Phosphogluconate dehydrogenase (PGD) - This dimeric locus gave single banded homozygotes and three banded heterozygotes. Resolution was greatly

Table 3.3**Overlay staining solutions**

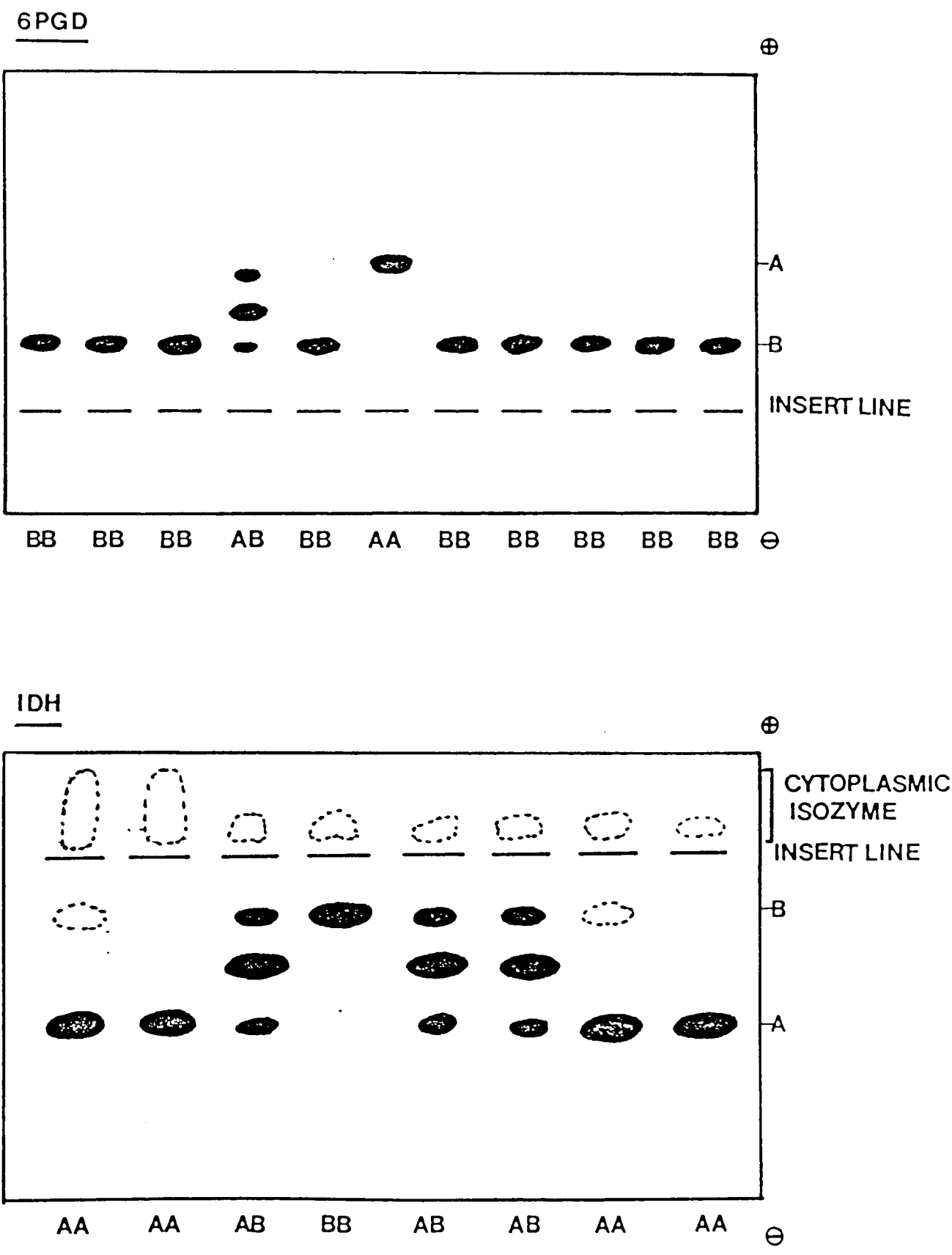
REAGENT	IDH	PGD	PEPD	PEPT
Buffer	6ml 0.5M Tris pH 8.0	8ml 0.2M Tris pH 8.0	8ml 0.2M Phosphates pH 7.0	8ml 0.2M Phosphates pH 7.0
Enzyme Substrate	30mg Isocitric acid (Na ₃ salt)	15mg 6-Phosphogluconate (Na ₃ salt)	20mg Leucyl-tyrosine	10mg Leucyl-glycyl glycine
Peroxidase 5mgml ⁻¹			1.0ml	1.0ml
L-Amino acid oxidase 4mgml ⁻¹			1.0ml	1.0ml
3-Amino-9-ethyl-carbazole 8mgml ⁻¹			1.0ml	1.0ml
0.2M MgCl ₂	1.0ml	1.0ml		
NADP 4mgml ⁻¹	1.0ml	1.0ml		
MTT 10mgml ⁻¹	1.0ml	1.0ml		
PMS 2mgml ⁻¹	1.0ml	1.0ml		
2% Agar @ 70°C	12.0ml	12.0ml	12.0ml	12.0ml
Total volume/gel	22.0ml	24.0ml	23.0ml	23.0ml

N.B. Footnotes for overlay staining solutions table (3.3)

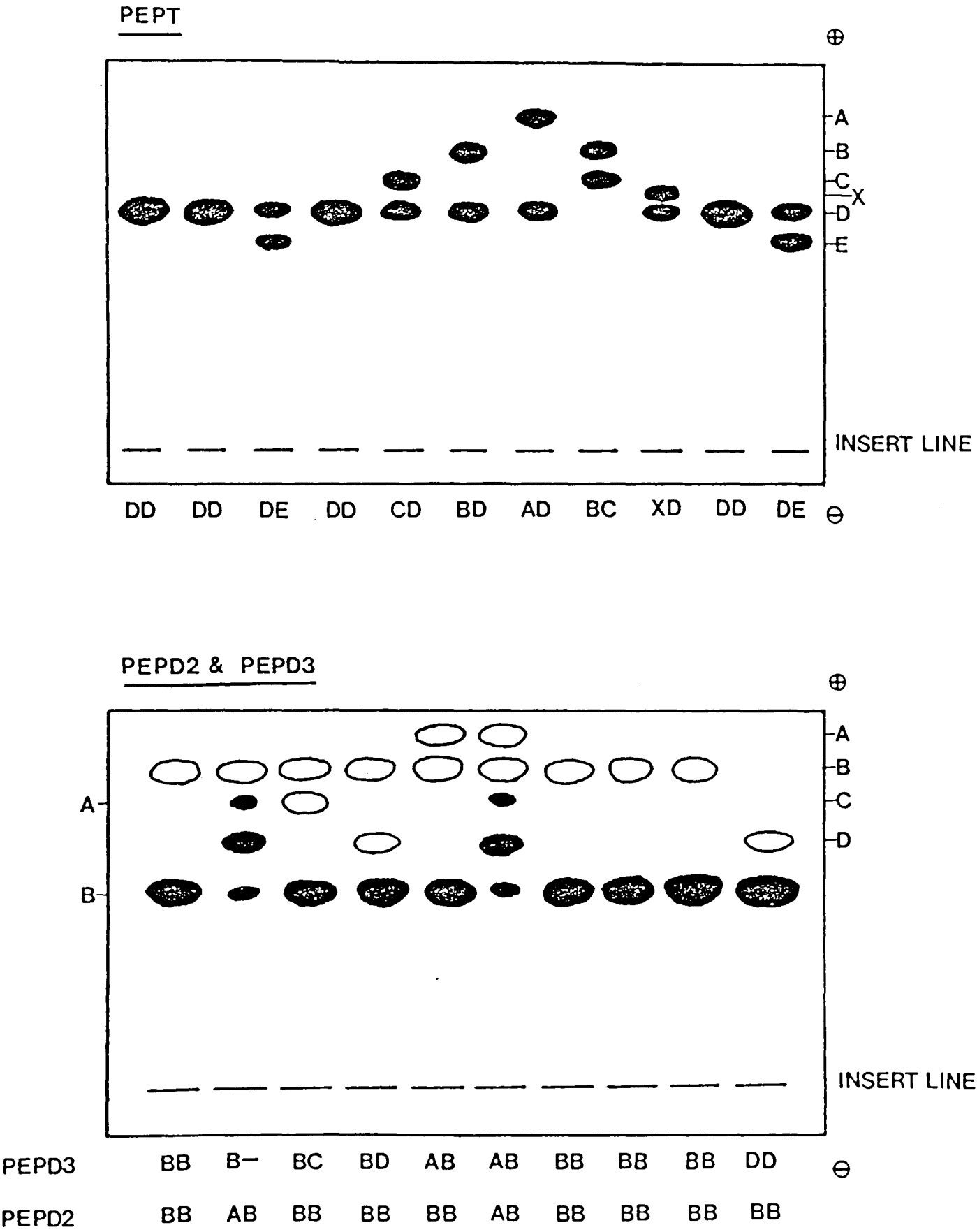
- 1) Peroxidase and amino acid oxidase are dissolved in 0.2M phosphates pH 7.0.
- 2) 3-Amino-9-ethyl carbazole is dissolved in acetone and added slowly to the staining mixture whilst stirring.
- 3) Leucyl tyrosine is dissolved one hour prior to use due to its low solubility.

Figure 3.1

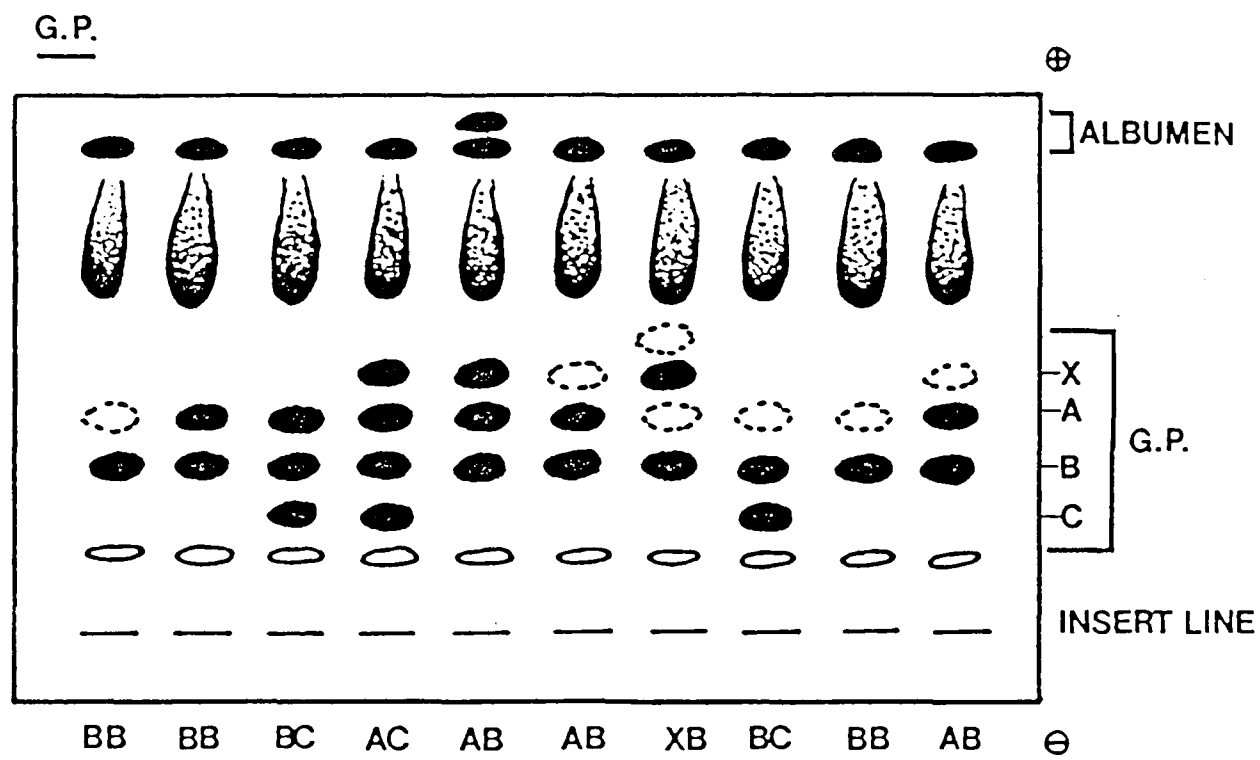
Diagrammatic representation of zymograms revealed by staining for 6PGD and IDH



Diagrammatic representation of zymograms revealed by staining for peptidases



Diagrammatic representation of zymograms revealed by general protein staining



improved by the addition of 20 mg of NADP to each gel just prior to degassing and a similar quantity to the cathodal buffer tank (Shaw and Prasad, 1970).

Isocitrate dehydrogenase (IDH) - also dimeric. This locus suffered from slight sub-banding which in the case of AA homozygotes resulted in an artefact band at the position usually occupied by the B allozyme. Heterozygotes were clearly distinguishable by the strongly staining heterodimeric band between the two homodimeric forms. Activity was also noted anodal to the insert line but was rarely scorable, the locus responsible codes for the cytoplasmic form of the enzyme whilst the scored locus is mitochondrial.

Peptidases (PEPD2, PEPD3 and PEPT) These loci were all scored from the same gel. One half was stained with the tripeptide Leucyl-glycl-glycine as substrate the other half with Leucyl-tyrosine. All suffered from post-translational modification resulting in anodal smearing which was corrected by incubation with DTT (see above). PEPD3, a monomeric enzyme migrated anodally to PEPD2 which is dimeric. PEPD2 AB heterozygotes obscured the positions occupied by the PEPD3 C, X and D alleles and so the genotype at this locus could not be scored in PEPD2 heterozygotes. PEPT is monomeric with simple two banded heterozygotes.

General Protein (GP). The variable locus revealed by this stain was identified as transferrin by Burke (1984) by comparison with published gel patterns for conalbumin and serum transferrin which are coded by the same locus (Ogden *et al.* 1962). GP homozygotes have a 2-banded phenotype, the anodal sub-band varied in intensity but never exceeded that of the slower band. Heterozygotes were either 3- or 4-banded depending on the genotype. The sub-band from the C allele comigrates with the B allele and similarly for B sub-bands and A alleles, thus AB and BC heterozygotes appeared with 3 bands whilst AC heterozygotes were 4-banded. This locus was particularly sensitive to temperature, poorly stored samples had more intense sub-bands

and stained more weakly. If the sample had lysed prior to separation of the plasma fraction the release of a multitude of cellular proteins made scoring of GP gels impossible.

Albumin was also found to be variable with a rare fast allele, the inheritance of which was confirmed in three families at Brackenhurst. Only a single variant phenotype was reported by Burke. This locus was not examined further due to the low frequency of the rare allele.

Burke's findings

It is always desirable to confirm the connection between an individual's zymogram and its assigned genotype. Burke attempted this using 357 nestlings whose putative parents he had identified by observation. Of these 12.9% possessed genotypes incompatible with the attendant adults. These discrepancies could arise either as a consequence of the birds' behaviour or through laboratory error. In the former case the attendants are not the true parents of the nestling which is the product of an extra-pair copulation, egg dump or an undetected mate change before the period of observation. Errors may be due to mis-identification of the feeding birds, mis-labelling of tubes or mis-scoring of zymograms. Some errors such as the first two mentioned will mimic the effects of falsely assigning parentage whilst the latter can only affect the single locus concerned.

The relative importance of the bird's behaviour and certain types of laboratory error in contributing to mismatches can be estimated from their different characteristics. Errors which associate the wrong blood sample with a bird can result in mismatches at more than one locus whereas mis-scoring of an individual's zymogram will not. Burke simulated the effect of incorrect parental assignment by reassigning adults at random

and recording the proportion of nestlings that mismatched at one or more loci. He also calculated exclusion probabilities for each locus using the method of Gundel and Reetz (1981). Exclusion probabilities are discussed in more detail later. Essentially, they estimate the proportion of young which will mismatch with incorrectly assigned parents given known allele frequencies within the population. The more alleles and the higher the heterozygosity at a particular locus the greater the probability that the true and incorrectly assigned parents will differ in genotype increasing the likelihood of detecting mismatching nestling zymograms. The results of both of these analyses showed a deficiency of nestlings mismatching at multiple loci relative to expectation. Burke concluded that this was due to mis-scoring of zymograms. However, the relative proportions of nestlings mismatching at each locus corresponded closely with the expected distribution implying that errors were more common at the most variable loci, which have the highest exclusion probabilities.

From these and other analyses, Burke estimated that about 70% of mismatches were caused by lab error. The remainder represented a non-paternity rate of approximately 6%. The confidence limits associated with the estimates are large because of the limited number of exclusions. The combined exclusion probability for all 6 loci was 51% implying that nearly half of the incorrectly assigned nestlings would have remained undetected. The inclusion of these nestlings may have affected both segregation and linkage analyses, e.g. a heterozygous male (A_1A_2) repeatedly cuckolded by an A_1A_1 male will apparently transmit the A_1 allele more frequently than expected. Similarly if the cuckolded is heterozygous at two loci A_1A_2, B_1B_2 and the cuckold is A_1A_1, B_1B_2 then there would be no evidence of non-random segregation between the loci even if they were in tight linkage disequilibrium.

The following sections will discuss the results obtained from samples collected at Brackenhurst subsequent to Burke's study. To avoid the problems of unidentified

cases of non-parentage the analyses are restricted to 370 parent-offspring trios sampled between 1985 and 1988 which have been confirmed by DNA fingerprinting.

Segregation

The nestling sample sizes for each locus are less than 370 because some bleedings yielded insufficient volume to score all six loci and provide DNA for fingerprinting. The total for PEPD3 also excludes all PEPD2 heterozygotes due to scoring difficulties (see above).

Segregation of heterozygous parental alleles was examined for each combination of parental genotypes. None deviated significantly from a 1:1 ratio (G-test, goodness of fit, Table 3.4) and so the data were pooled to combine all carriers of the same allele (Table 3.5). In this case only the C allele at GP approached significance ($G = 3.83$, $P < 0.06$), although the sample size was small ($N = 11$).

Interestingly, errors were confirmed in the scoring of zymograms at two loci, of which all but one involved the C allele at GP. Four nestlings from two broods produced by BB homozygous parents appeared to possess a band migrating at the expected position of a C allele. These individuals were repeatedly scored as mismatching at GP. The possibility of mis-labelling the plasma sample tubes on the day of collection can be excluded as no other sample from those days possessed the appropriate genotype. These findings cast doubt on the confidence with which GP can be scored, particularly with reference to C alleles. Since the phenotype is scored from a gel stained for all abundant serum proteins it is difficult to ascertain whether the aberrant bands arise by post-translational modification of transferrin, or from differential expression or modification of the product of another locus.

Table 3.4

Classification of offspring by their own and their parents genotypes.

The deviation from a 1:1 segregation was examined with a G Test for all pairings pooled within the same mating type. Five offspring confirmed by fingerprinting possessed phenotypes incompatible with their parents (shown in brackets). These individuals are discussed in the text.

		OFFSPRING GENOTYPE							
Locus (N)	Mating Type M F	AA	AB	AC	BB	BC	BD	CC	Devia- tion
6PGD (350)	AB x BB		11		5				N.S.
	BB x AB		18		22				N.S.
	BB x BB				294				-
PEPD2 (356)	AB x BB		13		12				N.S.
	BB x AB		9		15				N.S.
	BB x BB				307				-
PEPD3 (306)	AB x BB		5		2				N.S.
	BB x BB				218				-
	BB x BC				1	2			N.S.
	BC x BB				8	9		(1)	N.S.
	BB x BD				15		10		N.S.
	BD x BB				20		15		N.S.
IDH (355)	AA x AA	73							-
	AA x AB	27	27						N.S.
	AB x AA	37	30						N.S.
	AA x BB		5						-
	BB x AA		30						-
	AB x AB	25	43		22				N.S.
	AB x BB		4		10				N.S.
	BB x AB		13		9				N.S.

Table 3.4 (cont)

		OFFSPRING GENOTYPE							
Locus (N)	Mating Type M F	AA	AB	AC	BB	BC	BD	CC	Devia- tion
GP (348)	AA x AA	2							-
	AA x AB	7	8						N.S.
	AB x AA	3	3						N.S.
	AB x AB	12	31		15				N.S.
	AB x BB		45		49				N.S.
	BB x AB		5		13				N.S.
	AA x BB		5						-
	BB x AA		5						-
	AA x CC			10					-
	BB x BB				118	(4)			-
	BB x AC		2			0			N.S.
	BC x AB		3	0	0	0			N.S.
	BC x BB				5	3			N.S.
Locus (N)	Mating Type M F	AD	BC	BD	CD	CE	DD	DE	Devia- tion
PEPT (358)	AD x DD	2					0		N.S.
	BC x DD			0	2				N.S.
	BD x CD		3	0	0		3		N.S.
	BD x DD			23			29		N.S.
	DD x BD			8			6		N.S.
	DD x CD				15		12		N.S.
	DD x DD						237		-
	CD x DE				5	2	5	3	N.S.
	DE x DD						2	1	N.S.

Table 3.5

The number of offspring inheriting each parental allele from heterozygous parents. Segregations were tested for deviation from a 1:1 ratio

Locus	Parental Allele	Parental Allele Inherited	Alternative Allele Inherited	Goodness of Fit
GPGD	A	29	27	N.S.
	B	27	29	N.S.
PEPD2	A	22	27	N.S.
	B	27	22	N.S.
PEPD3	A	5	2	N.S.
	B	46	41	N.S.
	C	11	9	N.S.
	D	25	35	N.S.
IDH	A	174	163	N.S.
	B	163	174	N.S.
PEPT	A	2	0	N.S.
	B	36	38	N.S.
	C	25	29	N.S.
	D	73	67	N.S.
	E	6	12	N.S.
GP	A	120	134	N.S.
	B	139	118	N.S.
	C	3	10	N.S.

Exposure of transferrin to repeated freeze-thaw cycles or high temperatures increases the intensity of anodal sub-bands relative to the main bands, but decreases the intensity of all transferrin bands relative to background staining. If the C allele were less thermostable it is conceivable that BC heterozygotes could be confused with BB homozygotes following poor sample treatment. Alternative explanations discussed and dismissed by Burke as potential sources of error included the action of modifier loci or age related changes in enzyme activity. A possibility not considered but which may be applicable to GP is differential expression of an unconnected locus, e.g. Immunoglobulin G (IgG) becomes massively abundant in the plasma following a severe infection. Any locus whose expression is controlled by extrinsic factors may have resulted in the spurious band as long as sufficient quantities of the gene product were induced.

Having confirmed that scoring errors were most frequent at the locus where segregation distortion was most apparent, it must be determined why the mismatch rate was not suspiciously high. More than one factor may have played a part. Firstly, all mismatching trios were rerun in both Burke's and the present study. In the vast majority of cases the genotypes were confirmed but a small proportion of errors were detected at this stage. If the mismatch arose due to the failure to score a parental C allele, this may have been detected on the rerun. However, an unscored C allele in a nestling would not have resulted in a mismatch (unless the parent was a C homozygote) and thus would never be retested. Unfortunately, samples collected prior to 1985 were not available for re-analysis and confirmed families collected after this which were segregating for the C allele were rare ($N = 5$ broods).

Burke's segregation analysis excluded nestlings mismatching at the locus in question but included some products of extra-pair fertilization which were genetically compatible with the attendant adults. The affect of their inclusion is dependent on the sex of the scored adult heterozygote. When the male was the proband, repeated

cuckoldry could lead to an apparent deficiency amongst the assumed progeny of the rare allele, which the majority of cuckolders would lack. Cases where the female was the proband for segregation analysis will be affected to a much lesser degree. Paternal alleles derived from cuckolding males will seldom include the rare allele but the attendant male is similarly unlikely to possess it. Therefore, its frequency amongst the progeny will be determined by the maternal segregation ratio virtually irrespective of the rate of non-paternity. Since distortion was found in both sexes, undetected cases of non paternity were not a major influence.

Although providing no evidence of segregation distortion, the 1985-88 data illustrate the uncertainties of scoring GP. Errors in the 1980-82 data set were implicated whilst the positive correlation between the number of mismatching nestlings and the exclusion probabilities (P_E) for each locus implies that errors were most common at loci with the highest P_E , (e.g. GP). Misidentification of the true parents due to cuckoldry could not account for the distortion in both sexes, but observational error may involve either adult and cannot be excluded without fingerprinting although all loci would be affected to some extent. Thus the true cause of the apparent segregation distortion in Burke's sample remains to be uncovered.

The situation is analagous at PEPD3 with segregation distortion in the 1980-82 sample but not in 1985-88. The locus was involved in the remaining erroneous exclusion from the starch gel data. An apparent C homozygote nestling was produced by a phenotypically BC male and BB female attendant. This trio may provide evidence of a null allele present at very low frequencies for which the female was heterozygous. However, null alleles cannot explain the uneven transmission frequencies at PEPD3 because parents carrying the allele would appear homozygous and be excluded from analysis. Other problems with scoring PEPD3 were noted, including the occasional obscuring of the PEPD3 D allele position by anodal smearing from PEPD2, and considerably weaker staining of PEPD3 with respect to PEPD2 especially in some

PEPD3 heterozygotes. Combined, these could result in the rare D allele being missed in some individuals, though it is again unclear why this did not result in an anomalously high mismatch rate.

The occurrence of the unusual phenomenon of segregation distortion in two out of seven loci in the initial sample followed by its absence in a sample collected shortly afterwards raises the possibility that both loci were being influenced by the same factor. If the phenomena had a genetic basis then the similarity might arise through linkage. This was examined by Burke who found no evidence of linkage between any loci although his sample included undetected cases of non-parentage which, as stated previously, can conceal even tight linkage. This problem is eliminated by examining the fingerprinted families.

Linkage

The 370 nestlings of proven parentage were produced by 92 mate pairs. Legitimate sibships range in size from 1 to 12 with a mean of 3.98 ± 0.204 . The small size of the majority of sibships means that sufficient data cannot be collected from single families to test the hypothesis of linkage between most pairs of loci, therefore families had to be combined. This was done using the sequential probability ratio test (Morton 1955, 1957). The method requires no knowledge of parental phase (in coupling or repulsion) and is unaffected by the presence of null alleles.

The test calculates the log of the relative odds (LODS) of the observed segregation occurring given a test recombination frequency (θ_1) between the two loci compared with complete independence ($\theta = 0.5$). When the true recombination frequency is 0.5, i.e. the loci are unlinked, the probability ratio is 1 and the LOD is 0. If they are linked the tested recombination frequency (θ_1) will give the largest LOD

score when it equals the true frequency. Conventionally, if the LOD value for a trial exceeds 3, then linkage was confirmed as this was thought to reflect odds of 1000:1 in favour of linkage (Morton, 1955). However, unlikelihood is much more likely than linkage because there are many chromosomes and so in humans a LOD score of +3 in fact represents odds closer to 20:1 in favour of linkage whilst scores less than -2 provide grounds for rejecting linkage (Lander, 1988).

LOD scores are tabulated for a variety of θ values corresponding to a range of sibship sizes and their possible segregation (Morton, 1955 and 1957). The sum of the LOD scores from the available families was calculated for a variety of θ values and linkage was rejected when the summed LODS declined below -2.

Families are informative if they include at least one parent heterozygous at the two loci. When both adults share the same genotype, offspring heterozygous at those loci are uninformative. Since the parental phase is unknown, at least two offspring are required for each family. The results are given in Table 3.6, close linkage ($\theta < 0.01$) can be dismissed between all but three pairs of loci. No informative matings occurred involving adults heterozygous for 6PGD and PEPD2, while insufficient offspring were produced in families segregating for 6PGD and PEPD3 or PEPT to draw any conclusions about linkage. In all cases where adults were heterozygous at PEPD2, PEPD3 was not scored except for four matings involving PEPD2 heterozygotes which were confirmed as PEPD3 heterozygotes as well from the genotypes of their offspring. These individuals all produced nestlings which were heterozygous for neither locus and others heterozygous for PEPD3 only thereby proving the two loci were not closely linked. These families were only included in the analysis of linkage between the two dipeptidases and were excluded from other tests involving PEPD3.

Evidence was obtained to refute close linkage between most loci including GP and PEPD3. This is not unsurprising since the House Sparrow possesses 38 pairs of

Table 3.6

An analysis of linkage using LOD scores for a variety of tested recombination frequencies (θ)

			θ			
	Informative families	Informative offspring	0.01	0.05	0.10	0.20
6PGD - P2	0					
- P3	1	2 *				
- IDH	11	34	< -2	< -2	< -2	-1.7406
- PT	2	4	-1.1100			
- GP	6	15	< -2	< -2	-1.1304	
PEPD2- P3	4 +	10	< -2	-1.5600		
- IDH	6	19	< -2	< -2	< -2	-0.8958
- PT	2	11	< -2	< -2	< -2	-0.9691
- GP	6	20	< -2	< -2	< -2	-1.1508
PEPD3- IDH	8	26	< -2	< -2	< -2	-1.1082
- PT	5	21	< -2	< -2	-1.1566	
- GP	8	23	< -2	< -2	-1.3018	
IDH - PT	14	52	< -2	< -2	< -2	-0.5230
- GP	19	52	< -2	< -2	< -2	-0.8060
PEPT - GP	9	37	< -2	< -2	< -2	-1.2890

* No segregation observed though number of informative offspring limited

+ See text

chromosomes (Bulatova *et al.*, 1972). Therefore the loci follow normal Mendelian principles and segregate independently.

Genotype frequencies

Previous electrophoretic studies of House Sparrows have found that gene flow, genetic drift and random mating are sufficient to explain population differentiation (reviewed in Parkin, 1987). Likewise Burke's (1984) analysis of genotypic frequencies at the Brackenhurst and Sutton Bonington sites revealed little evidence of temporal variation or deviations from Hardy Weinberg although some discrepancies were evident at GP. The GP "C" allele frequency in adults showed an anomalous peak in 1981 and significant differences between the adult and pullus age classes. However, the differences were opposite in nature at the two sites.

The genotypic data collected annually over 9 years provides an opportunity to confirm temporal stability and so the following analyses include the years 1980-82 (T.A.B.) as well as 1983-88 (electrophoresed by J.H.W.). Firstly birds were classified as adults, known juveniles or pulli depending on their age when first handled. Birds of unknown age which may have been less than 8 months old were excluded from the analysis. The sexes can only be distinguished with confidence in adults. Therefore, no attempt was made to subdivide the immature age classes by sex.

Categorization by age, sex and year produced 204 samples (Appendix 1). Clearly, the normal statistical levels of significance cannot be applied to 200 tests as ten samples are expected to achieve significance at the 5% level purely by chance. To avoid spurious results, significance levels were recalculated using Sidak's test for multiplicative inequality. Each sample could then be examined for goodness of fit to

Hardy Weinberg using a G-test (Sokal and Rohlf, 1981). The sample statistic (G) is approximated by the χ^2 value with the same degrees of freedom.

Heterogeneity between the sexes and sample years was examined with the familiar χ^2 test. For many comparisons in both tests, sample sizes in some categories are very low, the effect of which is to artificially inflate the value of the χ^2 statistic. Therefore columns containing cells with expected values less than 1 were excluded from tests when their inclusion would otherwise have increased the value of χ^2 above 3.84, below this value heterogeneity cannot be significant.

RESULTS

Allele frequencies

The data from all loci fitted well with Hardy Weinberg expectations. The few G_{HW} values which exceeded 4 were mostly distributed at random across loci, years and age classes. However some high G_{HW} values were not associated with low expected frequencies, e.g. the deficiency of IDH heterozygotes amongst nestlings in 1982, 1984 and overall. Burke found that the anomalous results at this locus were the result of non-independence of the nestlings comprising the sample. Within each year related pulli will originate from sibships of different sizes, the largest of which will have an inordinate effect on the allelic and genotypic frequencies. In order to gain a truer reflection of the allele frequencies in the pullus sample the effect of sibship size must be accounted for by weighting. This was attempted as in Burke's study using the methods of Cotterman (1947). The resultant weighted frequencies did not deviate from Hardy Weinberg but this may be a consequence of the reduced sample numbers.

The effect of non-independent sampling is dramatically demonstrated at GP where a significant deviation from Hardy Weinberg in the 1986 adults ($G_{HW} = 23.05$) is due solely to a CC homozygous female, when this bird is excluded the fit to Hardy Weinberg is perfect ($G_{HW} = 0.00$). The female paired with an AA homozygous male and together they produced ten AC heterozygote offspring which in turn distorted the nestling ratios in 1986 and 1987. Omission of the female and her offspring returns the data to Hardy Weinberg equilibria. Similarly all the PEPD2 AA homozygote nestlings sampled in 1983 originated from a single pair which contributed 75% of the AA homozygotes recorded throughout the study. Overall it was concluded that there were no deviations from Hardy Weinberg that could not be ascribed to non-independence.

The adult sample

The adults were examined for heterogeneity firstly between the sexes. Fifty four inter-sex comparisons were made, none of which produced a χ^2 value exceeding the critical value of 10.924 corresponding to the adjusted 5% significance level (Table 3.7). Therefore the sexes were pooled for the analysis of temporal variation. Of the six loci only GP showed nearly significant heterogeneity between years and this was largely due to an excess of "C" alleles as previously noted by Burke. The analysis was repeated using counts of alleles rather than genotypes and once again GP was the only locus to approach significance. Thus allele frequencies were relatively constant during the study period with the possible exception of the difficult to score "C" allele.

Parentage Analysis

The efficiency of the starch gel technique at detecting non-parentage was tested using all 420 fingerprinted nestling/adult trios which were known to include 51

incorrectly assigned males. Twenty-eight nestlings were incompatible with the enzyme genotypes of the attendant adults, three of these mismatched at two loci (Table 3.8). Incompatibilities were found at every locus, most occurring at the more polymorphic loci.

Eleven nestlings did not possess an allele present in one of their apparently homozygous attendants. However, exclusions of this nature are not considered conclusive evidence of non-parentage, because the adult may be heterozygous for a null allele which is inherited by the nestling. Indeed, one of the starch gel based exclusions, the only one excluding a female, occurred in a family group confirmed by fingerprinting. This case probably arose through the inheritance of a rare PEPD3 null allele from the mother.

Unambiguous exclusion occurs when a nestling fails to inherit either of the alleles possessed by a heterozygous parent and therefore can only occur when there are three or more scorable alleles at a locus. In fact none of the remaining ten exclusions met this criterion but all excluded the male. The preponderance of mismatches involving the male attendant (10:1, $p < 0.01$) would imply that non-parentage arises almost entirely through cuckoldry rather than egg-dumping even in the absence of DNA fingerprint data. The other mismatches occurred when nestlings possessed an allele present in neither of the adults.

Cases of cuckoldry will go undetected if the alleles inherited from the true father are shared with the attendant male. This becomes less likely with increasing number of alleles per locus and higher heterozygosities. The exclusion probability (P_E) which is the chance of detecting an incorrectly assigned parent, is maximized when the allele frequencies are equal. This condition is seldom met by enzyme loci but it is approached by alleles at some minisatellite loci (Balazs *et al.*, 1989). Generally protein encoding loci have low exclusion probabilities due to the paucity of allelic variation and the

Table 3.8

Summary of attendant/nestling mismatches detected by starch gel electrophoresis

Locus	Site	Date of Bleeding	Genotypes			Excluded Parent	Brood Statistics (Number)			
			Pullus	Male	Female		Eggs	Hatch	Fledge	EPO
IDH	BH35	170585	AB	AA	AA	-	4	4	2	1
	BH18	190785	AB	AA	AA	-	5	5	4	1
	BG32	230686	BB	AA	BB	M	4	4	4	2
	BG32	230686	BB	AA	BB	M				
	BH42	020786	AA	BB	AB	M	3	2	2	1
	BH28	060886	AB	AA	AA	-	4	3	3	3
	BH28	060886	AB	AA	AA	-				
	BH54	270587	BB	AA	AB	M	4	4	4	2
	BH41	240687	AB	AA	AA	-	4	4	3	1
	BH54	200687	BB	AA	AB	M	4	4	3	2
	BH54	200687	BB	AA	AB	M				
	BH20	310787	AA	BB	AB	M	4	1	1	1
	BG42	270788	AB	BB	BB	-	4	4	2	2
6PGD	BH20	290587	AA	BB	AB	M	4	1	1	1
PEPD2	BH08	220787	AB	BB	BB	-	6	4	2	2
	BH33	100887	AB	BB	BB	-	5	5	3	3
PEPD3	BH51	200588	AB	BB	BB	-	3	1	1	1
	BH49	300588	AB	BB	BB	-	3	3	2	1
	BH15	170585	CC	BC	BB	(F)	5	5	4	0
	BH28	060886	BD	BB	BB	-	4	3	3	3
	BH19	110886	BD	BB	BB	-	4	4	4	1
	BH54	270587	BX	BB	BB	-	4	4	4	2
	BH54	270587	BX	BB	BB	-				
	BG42	270787	BD	BB	BB	-	4	4	2	2

Locus	Site	Date of Bleeding	Genotypes			Excluded Parent	Brood Statistics (Number)			
			Pullus	Male	Female		Eggs	Hatch	Fledge	EPO
PEPT	BH37	190785	BC	DD	CD	M	6	5	4	2
	BH09	050687	BD	DD	DD	-	4	4	4	2
GP	BH21	080785	AB	BB	BB	-	4	2	2	1
	BH21	230686	AB	BB	BB	-	5	4	1	1
	BH21	230786	AB	BB	BB	-	4	2	2	1
	BH19	110886	AB	BB	BB	-	4	4	4	1
	BH09	050687	AA	BB	AA	M	4	4	4	2
	BH21	200787	AB	BB	BB	-	4	4	1	1

Three pulli mismatched with their attendants at two loci: BH28 (060886) IDH and PEPD3, BH54 (270587) IDH and PEPD3 and BH19 (110886) PEPD3 and GP.

skewing of allele frequencies. However, information gained from independently segregating loci can be employed to raise the probability of detecting cases of non-parentage. The combined probability of exclusion $P_E(C)$ is given by

$$P_E(C) = 1 - \prod_{i=1}^i (1 - P_i)$$

where P_i is the exclusionary probability associated with the i^{th} system.

Many procedures for estimating the exclusion probabilities associated with codominant multiallelic loci have been derived. Potentially the most flexible method is that of Chakraborty *et al.* (1988) which allows for different allele frequencies in males and females. This procedure was applied to make allowance for the slight difference in observed allele frequencies between the sexes.

The method assumes that the loci are unlinked, selection is not acting, mating is random and the female attendant is the true mother. The previous analyses suggest that these conditions are met. Firstly the frequency of each mother/offspring genotypic pair is calculated using the allele frequencies in the male and female gene pools (as shown in Table 3.9). For each pair the associated exclusion probability is then calculated. These are then used to compute the average probabilities. An important point to note is that loci with high average exclusion probabilities will often give rise to mother/offspring pairs for which the exclusion probability is zero, e.g. for a diallelic system such as IDH any male can be the parent of a heterozygous nestling raised by a heterozygous mother.

Values of P_E range from 0.0241 (6PGD) to 0.1704 (GP). Low probabilities of detecting mismatches can lead to large sampling errors being introduced when extrapolating from observed mismatches. Hence estimates of EPF rate based on individual loci vary between 6.6 and 25.0% (Table 3.10). The $P_E(C)$ of 0.5454 is high enough to produce a realistic estimate of EPF rate for each year (Tables 3.11 and

Table 3.9

Allele frequencies determined from birds of known sex ringed post-fledge between 1985 and 1988

Locus	Sex	A L L E L E					N
		A	B	C	D	E	
IDH	M	0.719	0.281				363
	F	0.691	0.309				346
6PGD	M	0.026	0.974				365
	F	0.049	0.951				349
PEPD2	M	0.055	0.945				364
	F	0.046	0.954				349
GP	M	0.215	0.761	0.024			354
	F	0.205	0.773	0.022			339
PEPD3	M	0.014	0.891	0.023	0.070	0.002	327
	F	0.008	0.930	0.008	0.054	0.000	314
PEPT	M	0.001	0.037	0.021	0.925	0.012	365
	F	0.003	0.044	0.037	0.907	0.006	349

Table 3.10

Exclusion probabilities and EPF rates calculated using the data from
Tables 3.8 and 3.9

LOCUS	Exclusion Probability P_E	Observed Exclusion N_E	Tested Trios N_T	Estimated EPF Rate (%)
IDH	0.1589	12	402	18.8
6PGD	0.0241	1	397	10.5
PEPD2	0.0497	5	403	25.0
GP	0.1704	6	394	8.9
PEPD3	0.0650	5	334	23.0
PEPT	0.0740	2	407	6.6
Combined	0.5465	39	390	13.2

Table 3.11**Number of parent/offspring trios tested for genetic incompatibility**

Year	IDH	6PGD	PEPD2	GP	PEPD3	PEPT	P_E(C)
1985	70	76	76	71	57	76	0.5375
1986	127	125	127	123	108	127	0.5446
1987	155	154	155	151	131	155	0.5443
1988	50	42	45	49	38	49	0.5635
Total	402	397	403	394	334	407	0.5454

Table 3.12

Comparisons of EPF rate estimates from protein electrophoresis and DNA fingerprinting analysis

Year	Observed Exclusions		Estimated EPF rate (%)		No of finger-printed nestlings
	Enzymes	Fingerprints	Enzymes	Fingerprints	
1985	5	8	13.1	10.4	77
1986	7	14	10.5	10.6	132
1987	12	24	14.7	14.9	161
1988	4	5	15.6	10.0	50
Total	28	51	13.2	12.1	420

3.12). The estimated 13.2% of EPOs derived from the enzyme loci corresponds very closely with the 12.1% EPF rate from the fingerprinting survey.

The electrophoretic survey is a rapid and relatively cheap way of determining the source and extent of non parentage within a population when compared with DNA technology. However, the system has weaknesses: opportunities for laboratory error are greater as mis-scoring of a single zymogram will mimic non-parentage when the majority of individuals are expected to mismatch at only a single locus. The probability of a nestling mismatching at two loci is given by the product of their exclusion probabilities. Thus the P_E of a two locus mismatch is given by the sum of all pairwise products ($P_E \text{ double} = 0.1134$). The expected number of individuals mismatching at two of the electrophoretic markers is therefore only $51 \times 0.1134 = 5.8$ (3 individuals actually mismatched at two loci). Laboratory errors can and will occur and these artificially inflate the number of exclusions. The exclusions involving the C allele at GP were excluded from these analyses due to their confirmed unreliability.

Summary

Genetic variation at 6 protein coding loci was investigated using starch gel electrophoresis. The inheritance patterns of zymograms was shown to follow simple Mendelian patterns within families of confirmed parentage. No evidence was found of linkage between any loci although insufficient data were available to exclude the possibility of linkage between three pairs. All loci fitted closely to Hardy-Weinberg expectations and allele frequencies showed temporal stability.

Genetic incompatibilities between the nestlings and attendant adults were investigated to determine the power of starch gel surveys in parentage analysis. Errors were detected at two loci, a single case involved a putative null allele at PEPD3 but the majority involved the transferrin "C" allele which has previously shown segregation distortion and significant variation between years and age classes in frequency. Despite the uncertainty associated with this allele, mismatches involving the other alleles and loci occurred only in individuals known to be unrelated to the attendant male from fingerprint analysis. It was calculated from exclusion probabilities based on the estimated allele frequencies, that only 54.5% of cases of non-paternity would be detectable. The starch gel survey was fast, cheap and provided a good approximation to the true EPF rate (13.2% c.f. 12.1%). Reliability was high apart from the above mentioned exception, but its inability to detect all mismatches greatly reduces its power relative to DNA fingerprinting.

CHAPTER 4

DNA FINGERPRINTING

The development of minisatellite probes which can simultaneously detect several highly heterozygous multi-allelic loci has revolutionized the forensic determination of relatedness and identity. Several hypervariable loci had been identified previously (references in Jeffreys, 1987) but individually they offered little improvement over conventional protein electrophoresis and blood typing. Hypervariable loci share a common structure consisting of tandem repeats of 10 - 70 base pair units known as minisatellite sequences (Jeffreys *et al.*, 1985a). Between 6 and 80 alleles can occur at every locus each differing in the number of tandem repeats, hence the alternative name of minisatellite loci is VNTRs (Variable Number of Tandem Repeats) (Nakamura *et al.*, 1987).

The polycore probes developed by Alec Jeffreys consist of tandem repeats of the most highly conserved region (the core sequence) present in every repeat unit of a family of minisatellite loci (Jeffreys *et al.*, 1985a) (Fig. 4.1). The absence of the sections that show least homology between loci allow polycore probes to hybridize to an entire class of minisatellites in addition to the particular loci from which they were derived.

The analysis of minisatellite loci involves the basic techniques of molecular biology (Fig. 4.2). The sample DNA is initially digested with a restriction enzyme that recognises a 4bp sequence occurring frequently in the regions flanking the minisatellite loci but not within the repetitive region itself. The size of restriction fragments carrying minisatellites will be determined largely by the length and number of the repeated units. Electrophoresis through an agarose gel separates the fragments by size, after which their positions are permanently preserved by transferring the DNA onto a nylon membrane by Southern blotting (Southern, 1975). The filter is then hybridized with a radioactive minisatellite probe which binds to homologous genomic sequences. Excess

Figure 4.1

Minisatellite Structure

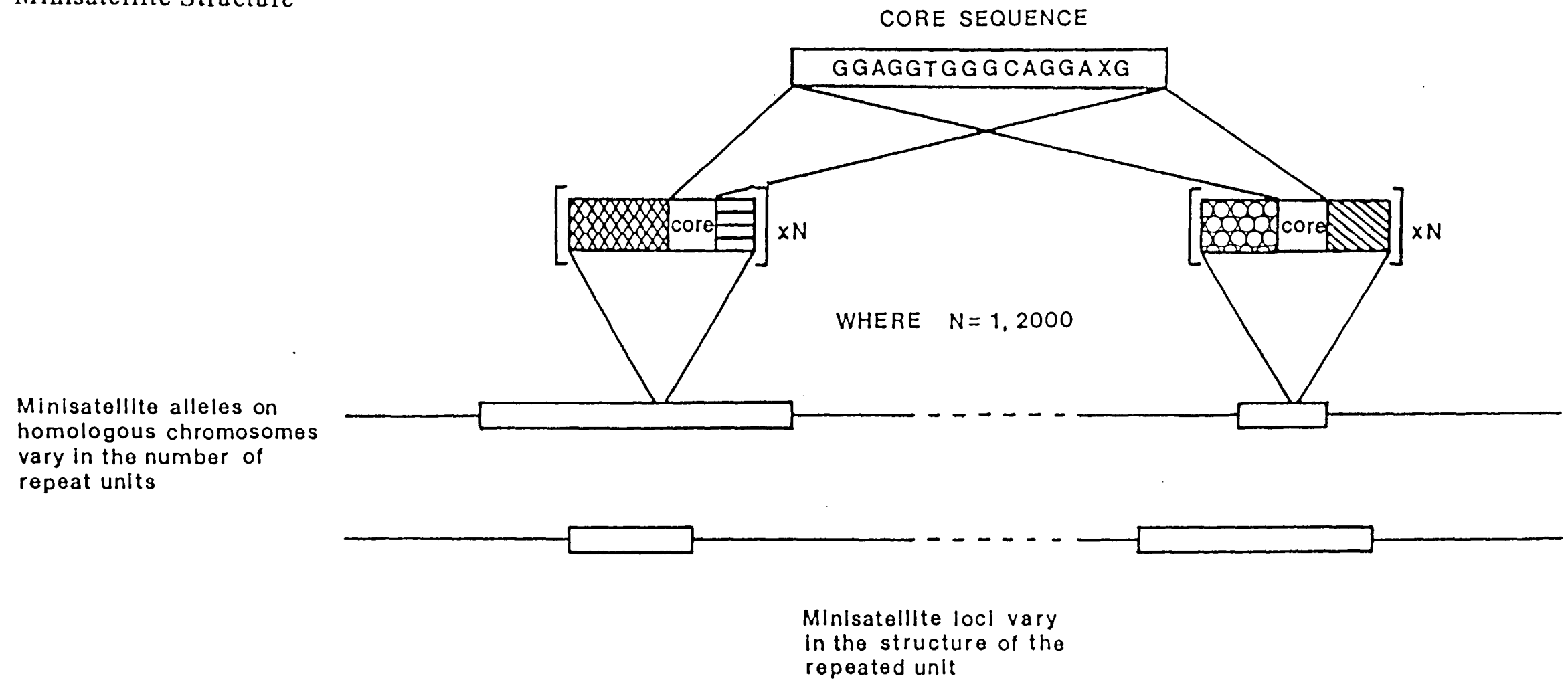
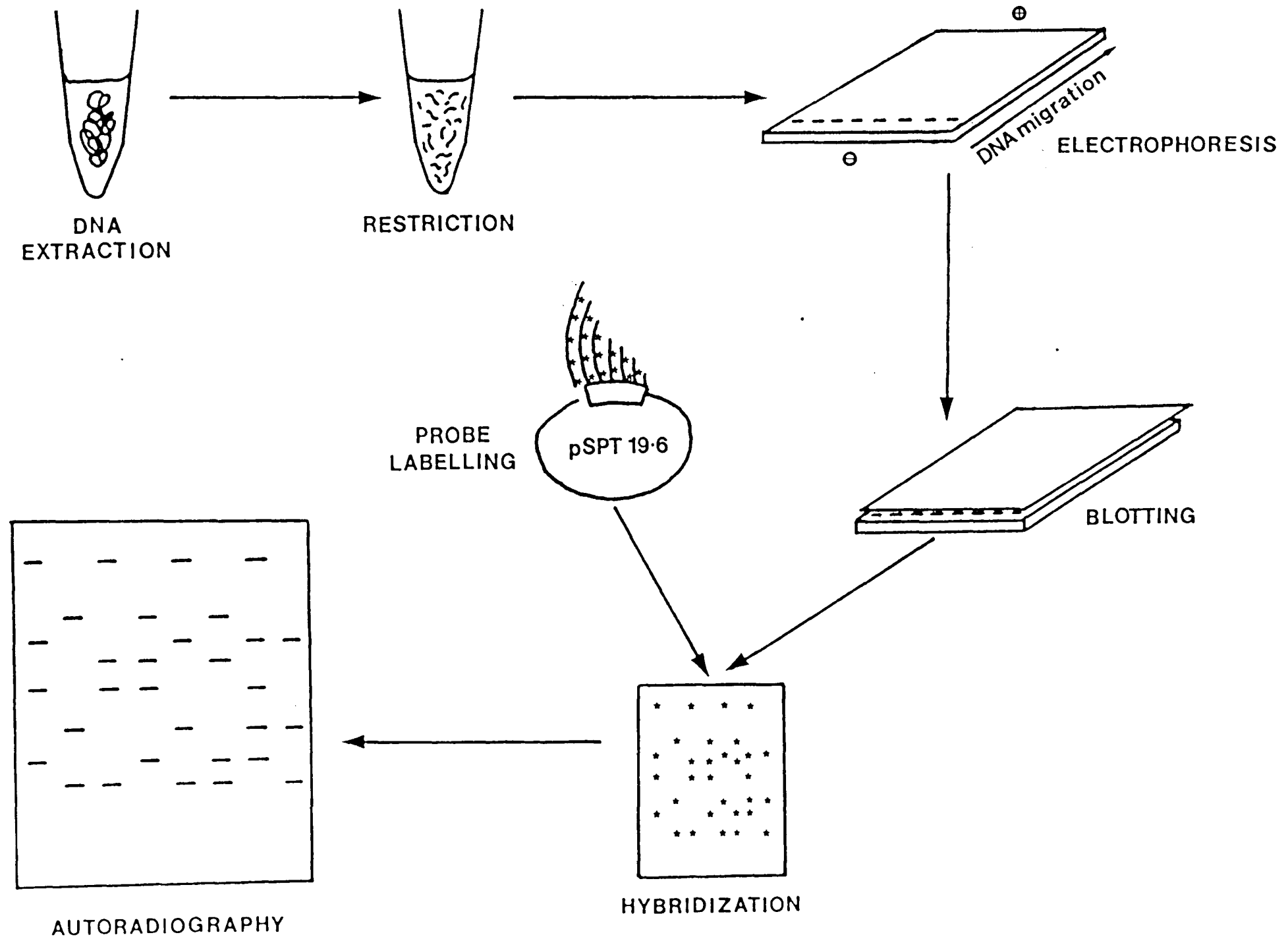


Figure 4.2

Outline of the DNA fingerprinting technique



probe is washed off prior to exposing an X-ray film to reveal bands where the probe has bound.

Individual bands in human fingerprints are derived from Mendelian loci and can be traced back to one or other of the parents. The majority are inherited independently but linkage and allelism do occur. Linkage arises when changes in the sequence of a single repeat unit create a recognition site for the enzyme so that the minisatellite bearing fragment is cleaved in two. If the recognition site was common to each repeat, then the locus would be degraded into individual subunits and lost from the gel. Close linkage between loci differing in repeat unit structure has been reported but is rare (Royle *et al.*, 1988). Allelism is also uncommon, suggesting that alleles vary greatly in size with some appearing in the scorable high molecular weight region of a gel whilst the alternative allele is lost in the mass of poorly resolved low molecular weight bands.

An important characteristic of minisatellite loci is the high rate at which new alleles are created. The similarity of the consensus core sequence to the χ recombination site in *E. coli* suggested that unequal recombination might generate new length variants. However, studies of individual human loci flanked by tightly linked markers has revealed that most changes consist of the loss or gain of a few repeats by sister chromatid exchange or meiotic slippage with no change in phase between the linked markers. The evidence for and against minisatellites as recombination hot spots was reviewed by Jarman and Wells (1989). Since the publication of Jarman's review, it has been shown that mutations at one human minisatellite locus at least arise solely through unequal sister chromatid exchange (Jeffreys *et al.*, 1990). As a consequence of this each allele evolves as an independent haploid lineage which can be identified by internal mapping (Jeffreys *et al.*, 1990) and used to trace more distant relationships than can be achieved by multilocus fingerprinting. "Mutation" rates vary greatly between loci, some of the highest being 0.01/gamete at the mouse locus M_S6hm (Jeffreys, Wilson *et al.*, 1987) and 0.052/gamete at the human locus λ MS1 (Jeffreys, Royle *et*

al., 1988). Heterozygosity is strongly correlated with mutation rate being dramatically higher at loci with heterozygosities greater than 95%, the relationship between the two fits closely to a model based on neutral mutation and random genetic drift (Jeffreys, Royle *et al.*, 1988).

The probability that an individual shares all its bands with another is given by x^n where n is the number of independently segregating bands and x is the probability that a band present in one individual is present in the next (Jeffreys, Wilson and Thein, 1985b). The combination of multiple alleles and high heterozygosity ensures that band sharing will be infrequent. The number of informative bands in humans is high because the two commonly used polycore probes 33.6 and 33.15 detect unrelated sets of minisatellite loci at which linkage and allelism are rare. For humans the probability x^n is 5×10^{-19} and the probability of identical patterns is very much lower still. The immense variability of human minisatellite patterns ensures that only identical twins will have identical patterns, hence the term "DNA fingerprints".

The demonstration of DNA fingerprints in other mammals including the cat, dog (Jeffreys and Morton, 1987), and mouse (Jeffreys, Wilson *et al.*, 1987) has been followed by the detection of homologous regions in organisms as diverse as *Tilapia* (Carter *et al.*, in press), an aphid (*Myzus persicae*) (R.E. Carter, pers. comm.) and rice (Dallas, 1988). The widespread evolutionary conservation of minisatellite sequences suggests that many of the uses to which fingerprinting has been put in humans can be equally applicable in other organisms. With this in mind, DNA fingerprinting was developed as a demographic tool to examine the breeding system of the Brackenhurst population.

The following sections describe the techniques involved in producing House Sparrow minisatellite patterns, an analysis of their inheritance and a discussion of the distribution of EPOs revealed by the fingerprinting survey.

METHODS

The experimental procedures involved in the production of a DNA fingerprint are summarised in Fig. 4.2, detailed protocols of each stage follow. Some refinements were made during the course of the study: in general, these modifications were minor but the change from DNA to RNA probes precipitated changes in other stages including blotting and hybridization conditions. Brief details of the earlier methodologies are given in Appendix 4. A variety of buffers and solutions are used, the recipes of which are given in Appendix 3.

DNA Extraction

DNA is extracted from 15 μ l aliquots of whole blood resuspended in 0.65ml of 1 x SET on the day of collection and subsequently stored in a 1.5ml eppendorf at -80°C until required. The following protocol is used:-

- 1) Thaw sample, add 15 μ l of 10mg/ml Proteinase K stock solution, mix gently, add 7.5 μ l of 25% SDS, mix gently and incubate overnight in a 55°C waterbath.
- 2) Prepare phenol solution by dissolving an equal volume of phenol crystals in 1M Tris pH 8.0. Transfer half of the phenol solution to a fresh container and add an equal volume of chloroform/iso-amyl alcohol (23:1, V:V), phenol/chloroform separates out as the lower layer.
- 3) Add 0.5ml phenol solution to the sample and mix gently by repeated inversion on a rotary mixer for 30 minutes.

- 4) Centrifuge sample at 8000g for 7 minutes to separate the aqueous and phenol layers. Proteins and cellular debris precipitate at the interface.
- 5) The upper aqueous layer is transferred to a fresh tube using a 1ml disposable plastic pipette tip with the end snipped off to increase the bore. (This reduces damage to the DNA which can become sheared by repetitive passage through narrow apertures.) Ensure that none of the interface is carried over.
- 6) Repeat stages 3 - 5 increasing the volume of the aqueous layer to 0.5ml with 1 x TE at stage 3. The duration of mixing can be reduced as the solution clears. Phenol extraction is repeated until the aqueous layer is colourless, this may require up to three changes.
- 7) Repeat steps 3 - 6 twice, substituting phenol/chloroform solution for phenol.
- 8) Repeat stages 3 - 5 once, substituting chloroform/iso-amyl alcohol (23:1, V:V) for phenol.
- 9) Transfer aqueous layer to a fresh eppendorf and slowly add 950µl of cold (-20°C) absolute ethanol. DNA starts to precipitate at the interface. Swirl tube vigorously to mix the two layers thoroughly. The DNA precipitate forms a fluffy white mass.
- 10) Pellet DNA by centrifugation at 8000g for 7 minutes, pour off ethanol and replace with 75% ethanol. Agitate tube to free pellet, centrifuge briefly and pour off ethanol, remove remaining liquid with a disposable pipette tip.
- 11) Dry DNA in vacuo or in a 37°C incubator for 15 minutes.

12) Resuspend DNA in 150 μ l of TE by overnight incubation in a 55°C waterbath.

Samples were processed in groups of 20 to 50 a day. On completion of extraction, tubes were labelled with the BTO ring number and stored in a cold room at 4°C until required for restriction analysis.

DNA restriction and assay

15 μ l aliquots of resuspended sparrow DNA were cut with 1 μ l of Hae III (approximately 10 units depending on the manufacturer) in the presence of 4mM spermidine (2 μ l of 40mM stock solution) and 2 μ l of the appropriate 10 x reaction buffer. Digestion was carried out in a 37°C waterbath for a minimum of 4 hours, or more usually, overnight.

Minigels were prepared by microwaving 0.24g LE agarose with 30ml of 1 x TBE buffer in a 100ml pyrex flask until the slurry had dissolved completely. Distilled water was added to replace that lost through evaporation and the flask cooled to 55°C in a waterbath. The molten agarose was then poured into a Cambridge Bioscience minigel apparatus fitted with a perspex comb to form 16 wells at one end of the gel. At least 20 minutes were allowed for the gel to set before it was covered with 70ml of 1 x TBE + 5 μ l of 0.5mg ml⁻¹ ethidium bromide.

To assay the quality of the restricted DNA, 2 μ l aliquots of the restriction reaction were mixed with an equal volume of 2 x BPB loading buffer. The buffer contains two dyes so that the progress of the electrophoretic run can be monitored, and Ficoll, a very high molecular weight polymer which increases the density of the sample allowing it to displace the running buffer from the wells in the gel. After loading the samples with a pipette using disposable plastic tips, the fragments were separated by applying a current

across the gel at 80V for about 50 minutes. When the purple dye was 2cm short of the edge, the current was switched off and the running buffer emptied into a beaker of dilute bleach to break down the ethidium bromide which is a potent carcinogen.

Ethidium bromide contains a planar subunit that intercalates between the bases of the DNA helix. When exposed to UV light at 354nm from a transilluminator, it fluoresces orange allowing the distribution of fragment sizes to be visualized. Partially digested samples display an excess of high molecular weight fragments. In such cases an additional 2 μ l of restriction enzyme were added and the samples reassayed later by minigel. A deficiency of large fragments was indicative of nuclease contamination, a problem only encountered when DNA was extracted from the pellet of cellular debris in tubes used to prepare erythrocyte lysates for starch gel electrophoresis. Families including partially digested or degraded samples were omitted from the parentage analysis because of the possibility of artefact bands.

Initially, the concentration of DNA was estimated from the intensity of fluorescence in the minigel assay but estimates were inconsistent, particularly when samples were concentrated. The acquisition of a Hoefer TK-100 DNA fluorimeter greatly improved the repeatability of assays. 2 μ l aliquots of restriction digest were suspended in 2ml of 1 x TNE buffer containing 0.1 μ g ml⁻¹ Hoechst 33258 dye in a disposable plastic cuvette. The cuvette was inverted several times to disperse the DNA evenly before insertion into the machine. The dye binds DNA avidly and emits light at 458nm when excited by UV light at 365nm. The intensity of emitted light is measured by a photosensitive cell and converted to a digital reading which is linearly related to the concentration of DNA. The use of a reference sample of turkey DNA allowed all restrictions to be diluted to a concentration of 150 μ g ml⁻¹ of DNA with BPB loading buffer.

DNA electrophoresis

0.8% agarose maxigels were prepared by microwaving 3.00gm of LE agarose with 375ml of 1 x TAE in a screw top 500ml Duran flask. When completely dissolved, the flask was placed in a 55°C waterbath for 1 hour to cool and then poured onto a 24 x 20cm perspex gel base, sealed at each end with masking tape. A 16 tooth plastic comb, 2cm from one end, was used to form a row of wells as the gel solidified. After a further 2 hours were allowed for the gel to set, the masking tape was removed and the gel immersed in 2.625l of 1 x TAE in a LKB H4 horizontal electrophoresis tank. The comb was removed and 40ul of the diluted restriction digests loaded into each well.

The samples were left to equilibrate with the running buffer for 10 minutes before a voltage was applied. The gels were run at 30V for 18 hours followed by a further 48 hours at 42V. At the end of the separation, fragments smaller than 3.5kb had migrated off the end of the gel.

Blotting

When separation was complete the gel was carefully inverted, transferred to a plastic tray and soaked in 0.2M HCl for 20 minutes. The weak acid damages some purine bases leaving single stranded nicks in the helix. This reduces the size of the larger minisatellite bearing fragments sufficiently to permit efficient transfer from the gel.

After depurination, gels were immersed for 35 minutes in 1.5M NaCl, 0.5M NaOH which denatures the helix releasing single stranded fragments and then equilibrated in 1.5M NaCl, 0.25M NaOH (ATS) for 10 minutes prior to blotting. A modified Southern blotting technique was used to transfer the DNA onto an Amersham

Hybond-N nylon membrane.

The gels were trimmed by removing a 4cm strip from the well end, and placed onto a wick of Whatman 3MM paper supported on a plastic plate above a reservoir of ATS. A 20 x 20cm sheet of Hybond-N was placed onto the surface of each gel, followed by two 20 x 20cm squares of Whatman 3MM. Each layer was thoroughly soaked in ATS before placement and great care taken to avoid trapping air bubbles between the layers. Finally, a stack of dry absorbent paper towels (Kimberly Clark, UK) 10cm deep was layered on top of the blot and gently compressed by a 500g weight.

DNA transfers out of the gel onto the membrane as ATS is drawn through the gel from the reservoir into the towel stack. After 18 hours the blot is dismantled and the filter briefly rinsed twice in 2 x SSC to remove the ATS, then left to air dry on absorbent paper for 20 minutes. Filters were baked at 80°C in vacuo for 20 minutes to fix the DNA to the membrane prior to storage.

Preparation of ribo-probe substrate

The inserts from the Jeffreys' polycore probes were subcloned into pSPT18 and 19 transcription vectors as described in Appendix 5. pSPT 19.6 and pSPT 18.15 have the inserts from the polycore probes 33.6 and 33.15 orientated so that the G-rich strand is cis to the T7 promoter, thereby producing the hottest transcripts when labelled with $\alpha^{32}\text{P}$ CTP. Large quantities of the plasmids were produced using scaled up miniprep techniques from overnight cultures of *E. coli* DH1 in 100ml LB Amp broth. The following method adapted from Ish-Horowicz and Burke (1981) was used.

- 1) Spin down 25ml of culture in a 30ml plastic centrifuge tube at 6,000 rpm for 6 minutes in a Sorval SS34 rotor. Discard supernatant, add a further 25ml and repeat the spin.
- 2) Discard supernatant and resuspend pelleted cells in 1ml of "Miniprep" buffer (50mM glucose, 25mM Tris pH 8.0 and 10mM EDTA). Leave for 10 minutes.
- 3) Following steps are carried out at 4°C unless stated otherwise.
- 4) Add approximately 20mg Lysozyme (by spatula) and leave for 30 minutes.
- 5) Add 2ml of 0.2M NaOH, 1% SDS and mix gently. Leave at room temperature for 5 minutes.
- 6) Add 1.5ml of precooled 5M potassium acetate (3M potassium acetate and 2M ethanoic acid; pH 5.6). Mix gently and keep on ice for 5 minutes.
- 7) Centrifuge at 10,000 rpm for 10 minutes.
- 8) Transfer supernatant and add to it an equal volume of phenol/chloroform, mix for 5 minutes. Spin at 10,000 rpm for 6 minutes.
- 9) Transfer top aqueous layer and repeat the phenol/chloroform extraction.
- 10) Transfer aqueous layer and wash with an equal volume of chloroform. Spin at 10,000 rpm for 6 minutes.
- 11) Transfer top aqueous layer and add 2 x volume of cold (-20°C) ethanol. Mix gently and leave overnight at -20°C.

- 12) Spin at 10,000 rpm for 10 minutes.
- 13) Remove the supernatant and wash the pellet with 70% ethanol, discard ethanol and draw off remaining liquid with a disposable pipette tip.
- 14) Dry pellet at 37°C for 20 minutes. Resuspend in TE overnight at 55°C.

Ribo-probe labelling

50µl aliquots of the resuspended plasmid were restricted with EcoRI (pSPT 19.6) or Hind III (pSPT 18.15). These linearize the plasmids by cutting the polylinker distal to the insert with respect to the T7 promoter. The digests were assayed fluorimetrically and diluted to 0.24µg µl⁻¹ of plasmid DNA with sterile TE.

Labelling reactions were set up using the following recipe:-

5.5µl restricted pSPT
1.0µl ATP (10mM)
1.0µl GTP (10mM)
1.0µl UTP (10mM)
4.4µl 5 x transcription buffer
2.0µl DTT (100mM)
1.0µl RNase inhibitor (25 units)
1.0µl T7 RNA polymerase (10 units)
5µl ³²P CTP (400 Ci mmol⁻¹)

The reaction was terminated after a 60 minute incubation at 38°C by the addition of 20µl of Nick-stop mix. Unincorporated nucleotides were separated from the labelled transcript by spun column chromatography. The column was formed inside the barrel

of a plastic 1ml syringe by placing a pad of glass wool at the bottom and then filling the barrel with TE buffered Biogel P60 granules. The column was packed by spinning at 2,000g for 5 seconds, then washed through twice with 150 μ l of TE by further 5 second spins. The flow-through was collected by a 15ml polypropylene tube in which the barrel was supported.

The probe was carefully layered onto the surface of the column, spun at 2,000g for 5 seconds then washed through with an additional 50 μ l of TE by a further 5 second spin. The Nick-stop mix contains two dyes, one comigrates with the unincorporated nucleotides and the other with the labelled transcript. The transcript is the first fraction to elute from the column. 1 μ l aliquots of the reaction before and after separation were mixed with Ecoscint scintillation fluid and assayed by scintillation counting to determine the efficiency of incorporation.

Prehybridization and Hybridization

Up to 16 20 x 20cm nylon membrane filters were prehybridized simultaneously in 500ml of 1 x SSC, 1% SDS, 1 x Blotto (Johnson *et al.* 1984) in a 22 x 22cm plastic box. The box was gently agitated in a 65°C shaking waterbath for 5 to 8 hours. The filters were removed briefly from the box while 6 x 10⁷cpm of the probe was added. The filters were returned ensuring that they were thoroughly immersed and no air bubbles were trapped. Hybridization was allowed to proceed overnight at 65°C.

Washing and Autoradiography

Filters were washed at a stringency of 1 x SSC, 0.1% SDS at 65°C with four changes of solution, each wash lasting 25 minutes. On completion of washing the

filters were air-dried on absorbant paper for 10 minutes, then, while still slightly damp, wrapped in Saran Wrap. The filters were initially autoradiographed using two calcium tungstate intensifying screens (either Cawo or Hi Speed X) with Fuji RX film at -80°C. An overnight exposure was used to determine the time required for a full exposure. If the signal was particularly intense, the intensifying screens were omitted to produce a sharper image. Screenless exposure lasted 3 to 10 days at room temperature.

Screening of gels

An automated scanning device with the power to discriminate between bands with the required level of precision was not available, so all autoradiographs were scored by eye. Parents were replicated at each end of a gel to aid scoring. The majority of gels showed little sign of distortion so that a ruler between the parental lanes would reveal the corresponding bands in the offspring. Where necessary, allowance could be made for gel distortion by identifying characteristic parental bands in nestlings. Slight distortion, variation in gel concentration and differences in the number of applied volt hours made comparison between gels virtually impossible for dissimilar samples, although corresponding bands from replicate samples of a single individual were readily identifiable.

Due to the difficulty of identifying corresponding bands between gels, it proved impossible to search for true parents of EPOs unless the individual was tested against all the potential fathers on the same gel.

Initially the radioactive probe was produced by nick translation of the double stranded RF M13 carrying the polycore inserts. This method produced probes of low specific activity, as much of the ^{32}P is incorporated in the vector (approximately 80%) rather than the insert. To achieve a visible signal from the autoradiograph, about 10ug

of genomic DNA had to be loaded into each lane. Overloading of the lanes caused pronounced curvature of the bands, a phenomenon known as “smiling”, which made precise comparisons of band position difficult. Even with overloaded lanes, exposure of up to 10 days with two intensifying screens were needed which, combined with a high level of non-specific background hybridization, resulted in fingerprints of poor quality. A variety of 4bp restriction enzymes were tested. Hae III gave the most evenly dispersed patterns between 5 and 30kb and preliminary tests on family groups revealed little evidence of non-random segregation, and band-sharing between unrelated Sparrows was less frequent than in humans (see Appendix 4).

The change to RNA probes eliminated the problems of smiling and reduced background noise. The probe is labelled by producing an mRNA of the most G rich strand of the insert. All the incorporated radionucleotides are present in the labelled transcript which binds avidly to homologous DNA and only 6µg of genomic DNA is required to produce a very intense signal negating the use of intensifying screens (see Fig. 4.3).

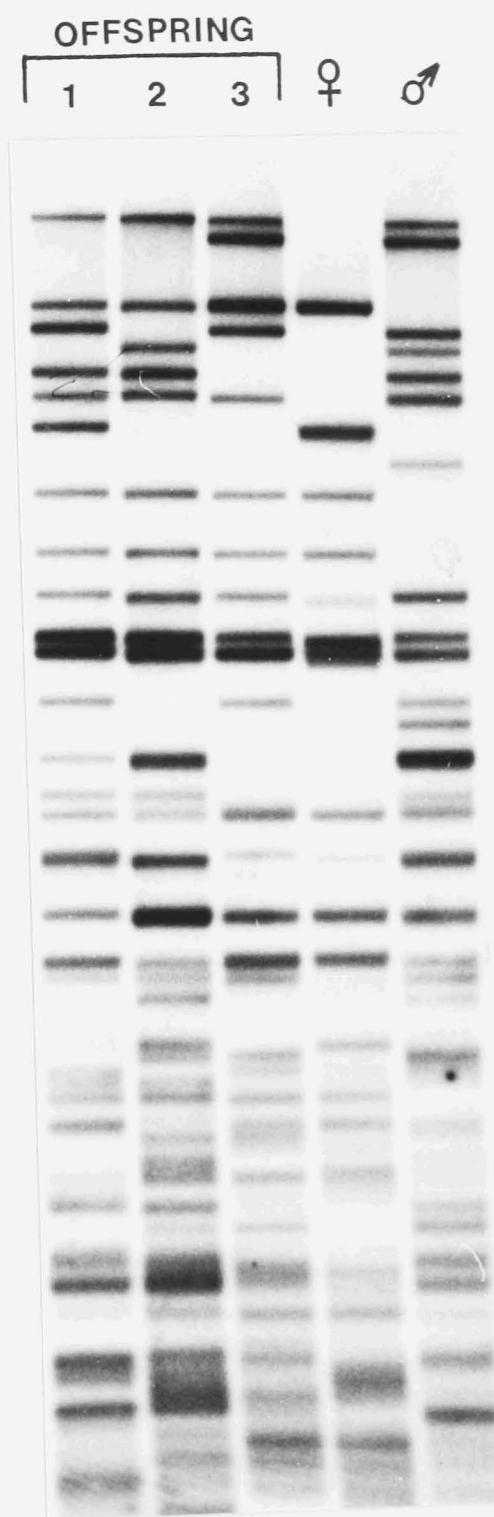
RESULTS

The Inheritance of Fingerprint Patterns

The results produced by the RNA probe were of sufficient quality to attempt a survey of all the families covered by the electrophoretic study. All nestlings were scored for the presence of mismatching bands that could have arisen either through EPF or mutation. This can be done quickly and repeatably whereas recording the segregation of every parental band within a family is very laborious and was restricted to 36 families in which both parents possessed at least 6 scorable bands larger than 5kb. Adults failing to meet this requirement usually did so because of inadequate separation of clustered bands rather than a lack of large fragments.

Figure 4.3

A simple House Sparrow Family



Each band present in the three offspring can be traced back to one or other of the parents demonstrating the simple inheritance of DNA fingerprints.

The mean number of scorable bands in adult males, females and pulli were 13.75 ± 0.568 , 10.72 ± 0.549 and 12.91 ± 0.288 respectively. The difference between the sexes was confirmed by comparison of mate pairs run in adjacent lanes ($t = 4.41$, $P < 0.0001$). This cannot be ascribed to an artefact of band resolution, and must reflect a difference in minisatellite complement between the sexes.

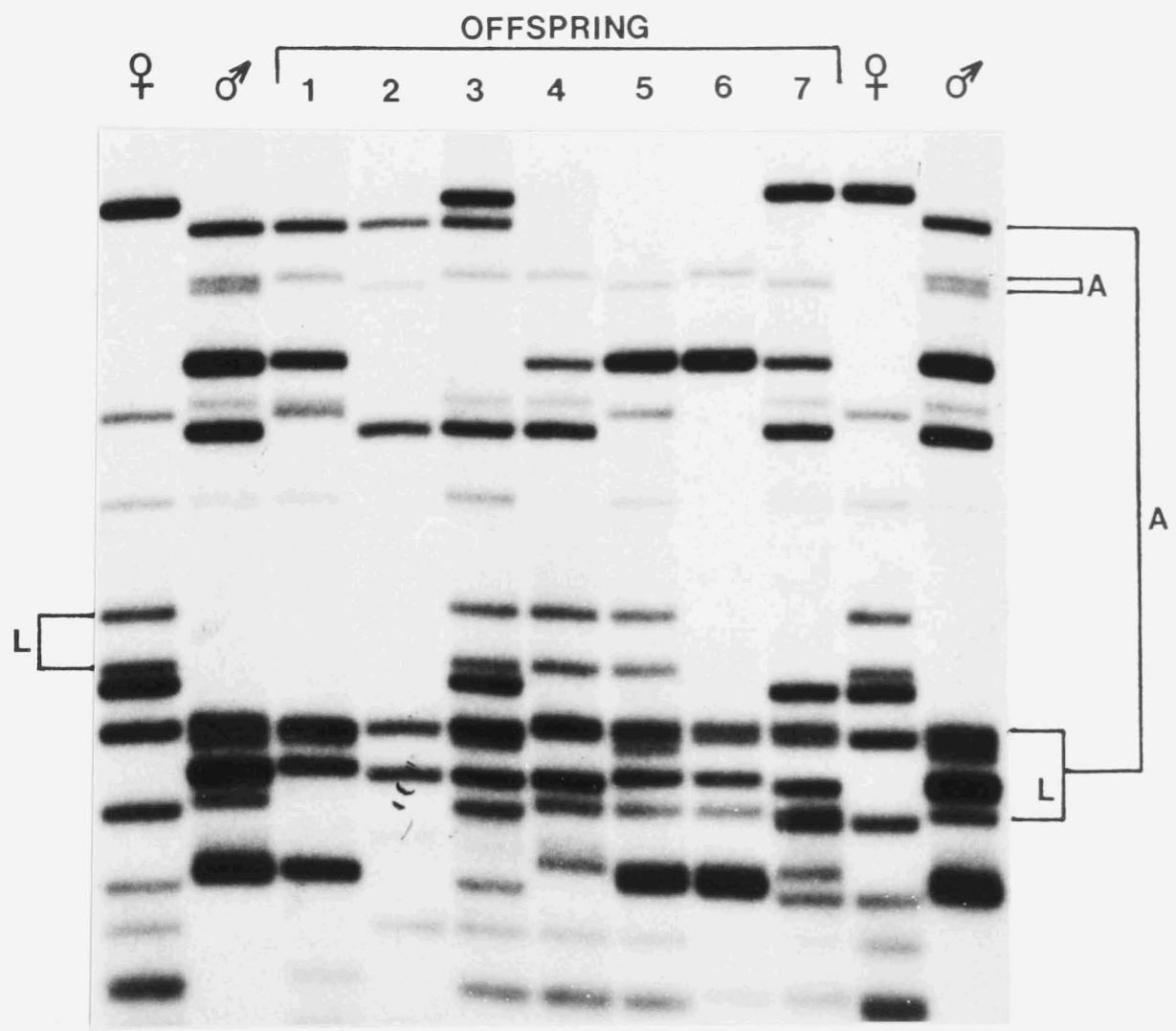
The nine largest full sibships consisting of 5 groups of 7 and one each of 8, 10, 11 and 12 offspring were examined for evidence of non-random segregation (for examples, see Figs. 4.4 - 4.6). The data for each family are presented in Table 4.1 and summarised by sex in Table 4.2. The adults which raised these large families presumably possessed minisatellite genotypes representative of House Sparrows in general. However, the band sharing between the parents of some of the larger families was unusually high for unrelated birds.

To test for non-random segregation of a pair of bands, both must be present in one parent and absent from its mate. The high degree of band sharing (approaching 50% for the parents of the largest sibship) limited the number of informative pairs and cases of non-independence may have gone unnoticed. Large sibships are required to reduce the risk of spurious associations between bands arising by chance. For example, in sibships of 7 individuals, 1 in 32 pairs of bands will yield false evidence of non-independence, but only 1 in 1,024 will be associated by chance in a sibship of 12. Binomial expectation predicts that in total 8.2 bands will show chance association within the five sibships of 7 compared with only 0.76 bands in the four larger families combined.

Linkage and allelism reduce the number of independently segregating bands that can be used for parentage analysis. 21.7% of paternal and 24.0% of maternal fragments cosegregated with another. The tight linkage disequilibria apparent in these families probably results from cleavage of repeat units containing Hae III sites to form a

Figure 4.4

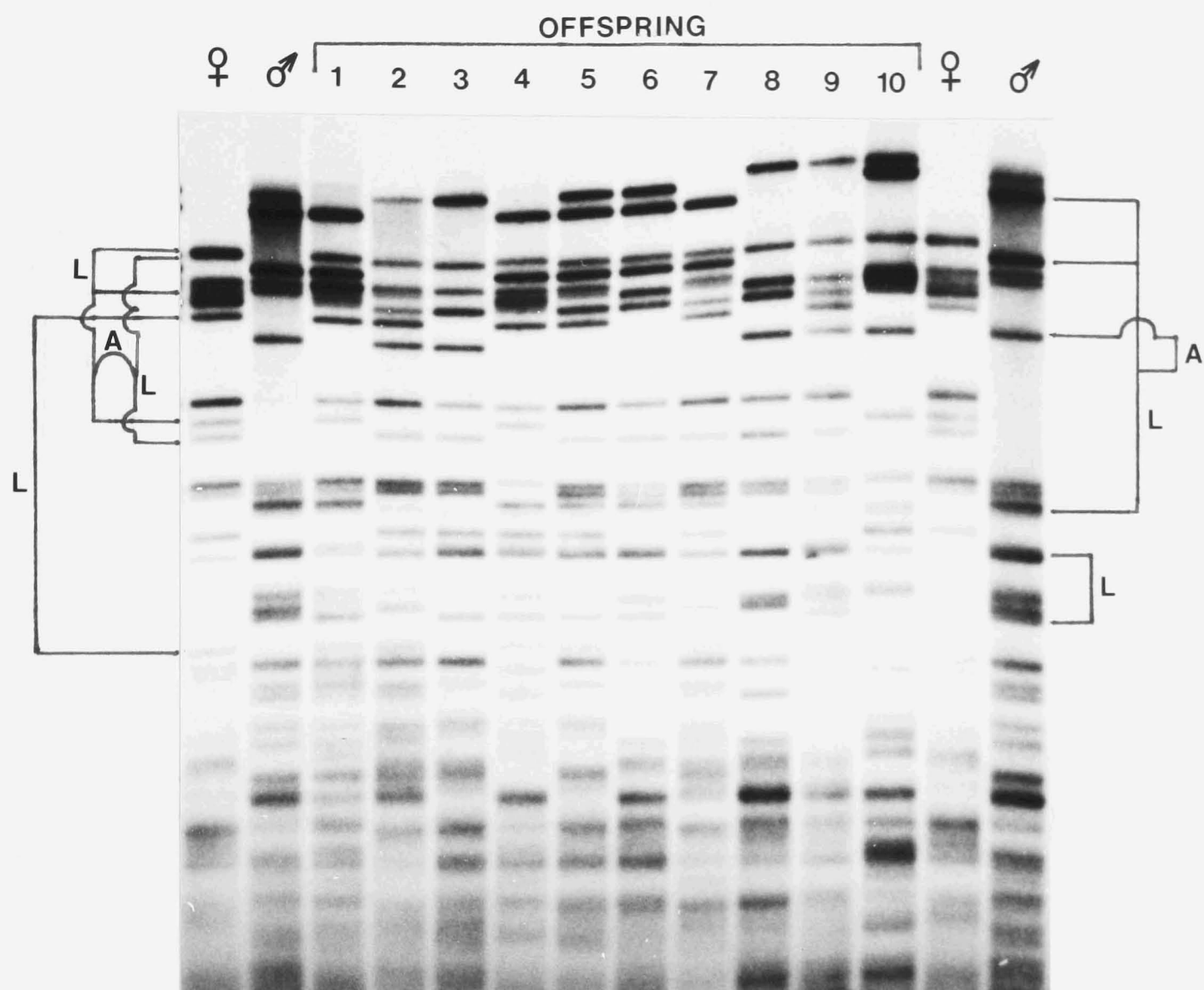
Possible examples of linkage and allelism in a House Sparrow family



Putative linked (L) and allelic (A) pairs of bands are joined. The female possesses a single linked pair, the male a linked pair and two sets of allelic bands. The non-independent segregation of these groupings were confirmed by examination of other offspring of the two adults.

Figure 4.5

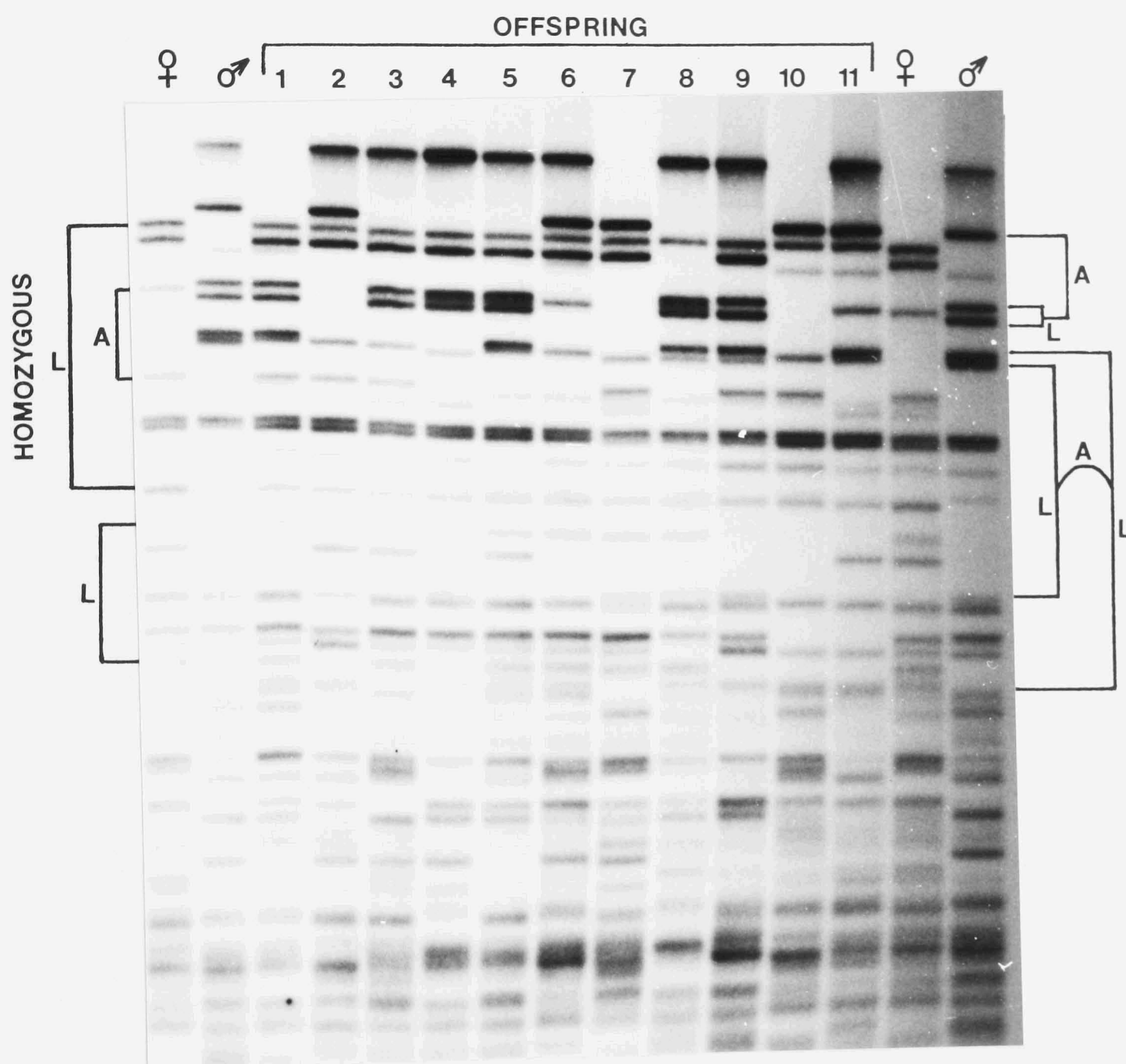
Family 7 in the segregation analysis



Possible cases of linkage (L) and allelism (A) are indicated.

Figure 4.6

Family 8 in the segregation analysis



Possible cases of linkage (L) and allelism (A) are indicated.

Table 4.1

The Inheritance of Fingerprint Bands in 9 Large Brackenhurst Families

FAMILY	1		2		3		4		5		6		7		8		9	
Parental Sex	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
Number of offspring	7		7		7		7		7		8		10		11		12	
No. of bands > 5kb	11	7	14	6	15	10	16	12	14	9	10	10	16	15	17	17	14	15
No. shared with mate	3	3	2	2	2	2	0	0	0	0	2	2	5	5	5	5	7	7
No. scored for segregation	8	4	12	4	13	8	16	12	14	9	8	8	11	10	12	12	7	8
Trans-mission frequency	44.7	53.6	48.9	50.0	53.9	35.7	51.4	44.7	48.3	50.0	53.1	36.0	51.8	58.0	53.3	52.7	66.7	43.9
No. linked to bands scored previously	3	1	2	0	1	2	3	5	6	1	0	3	3	4	3	2	1	0
No. allelic to bands scored previously	2	1	4	0	2	0	3	0	2	0	0	0	1	1	2	1	2	0
No. of informative bands	3	2	6	4	10	6	10	8	6	8	8	5	8	5	7	9	4	8

Table 4.2**Summary Statistics of Fingerprint Data from the Linkage Families**

PARENTAL SEX	MALE	FEMALE
No. of bands > 5kb	14.11 ± 0.77	11.22 ± 1.27
No. of allelic groups	2.00 ± 0.37	0.33 ± 0.17
No. of linked groups	2.44 ± 0.58	2.00 ± 0.58
Mean No. of bands in haplotype	2.22 ± 0.101	2.40 ± 0.221
No. of independently segregating parent specific bands	6.89 ± 0.81	6.11 ± 0.77
Transmission frequency	51.7%	47.5%

multi-fragment haplotype. Up to 4, but usually only 2, bands formed these haplotypes which did not differ in number of bands between the sexes ($P > 0.1$, Mann Whitney).

The proportion of allelic bands differed dramatically between the sexes. If all the bands in a fingerprint were derived from autosomal loci, allelic association would be equally frequent in both sexes. However, 18 of 21 recorded cases occurred in males, a significant difference ($P < 0.001$) even when allowance is made for the greater number of male bands. The discrepancy between males and females in the number of scorable bands is reduced when only independently segregating bands are considered.

Transmission frequencies were calculated for every band specific to one parent. Both sexes transmitted their bands to approximately half of their offspring (47.5% , $N = 75$ maternal and 51.7%, $N = 101$ paternal) suggesting that comigration of non-identical fragments is rare. Three apparently homozygous bands were not included in this analysis.

Common alleles are more likely to be shared by unrelated mates and be homozygous within individuals. Such bands will deviate from the expected 75% transmission frequency of shared heterozygous parental bands if one or both of the parents is homozygous. The expected probabilities of every offspring in sibships of the sizes examined in the segregation analysis inheriting the same shared parental band are given in Table 4.3. The observation that 12 out of 26 shared parental bands are inherited by every offspring confirms that a large proportion of shared bands are homozygous in at least one parent, and suggests that comigrating bands usually represent identical alleles or alleles that cannot be confidently resolved with the current methodology.

The fingerprints of all 420 nestlings were examined for mismatching bands. 199 nestlings were included in the 36 families in which the segregation of each parental

Table 4.3

Testing the segregation of shared parental bands.

Bands shared by both parents were assumed to be heterozygous in each. Many of these were found in every offspring. The probability that the observed or a greater number of shared parental bands would be present in every offspring was calculated using a binomial distribution with a 75% chance of each inheriting the shared band.

Sibship Size	7	8	10	11	12
Number of bands shared by parents	7	2	5	5	7
Number of bands shared by all offspring	3	1	2	3	2
Probability of observed segregation assuming each parent is heterozygous	0.0549	0.1902	0.0283	0.0007	0.0189

e.g. the probability that 3 or more of 7 shared parental bands would be present in all offspring in a sibship of 7 is given by:-

$$\begin{aligned} P &= 35 (0.75^{21}) (1 - 0.75^7)^4 \\ &+ 35 (0.75^{28}) (1 - 0.75^7)^3 \\ &+ 21 (0.75^{35}) (1 - 0.75^7)^2 \\ &+ 7 (0.75^{42}) (1 - 0.75^7) \\ &+ (0.75^{49}) \end{aligned}$$

band was recorded. These families included 24 nestlings with several mismatching bands > 6kb (mean = 4.88 ± 0.46) and three others, all unrelated, with a single mismatching band.

The 24 multiple mismatches probably arose through incorrect assignment of parentage. Each of these birds possessed mismatching bands in the unscored low molecular weight region, no such bands were present in the three individuals with single mismatching bands which were attributed to “mutation” (Fig. 4.7).

The 175 apparently “legitimate” offspring possessed between them 2,254 scorable bands of which 3 could not be assigned to a parent. The mutation rate was estimated as 0.0013/band or 0.017/bird. Both are conservative estimates as the remaining 221 nestlings included 27 other multiple mismatches but no cases of single mismatching bands. The true mutation rate to new alleles is thus slightly lower than the value of 0.004 found in humans (Jeffreys *et al.*, 1987a).

Mutations are unlikely to produce more than one scorable mismatching band. To do so would require the creation of a Hae III site within a fragment large enough to generate two scorable bands, a rare occurrence in comparison with the loss or gain of several tandem repeats from any position within a large minisatellite. The probability of two independent mutations occurring in the same bird is $(0.017)^2 = 0.0003$ which supports the contention that mutation cannot be responsible for the numerous cases of multiple mismatching bands.

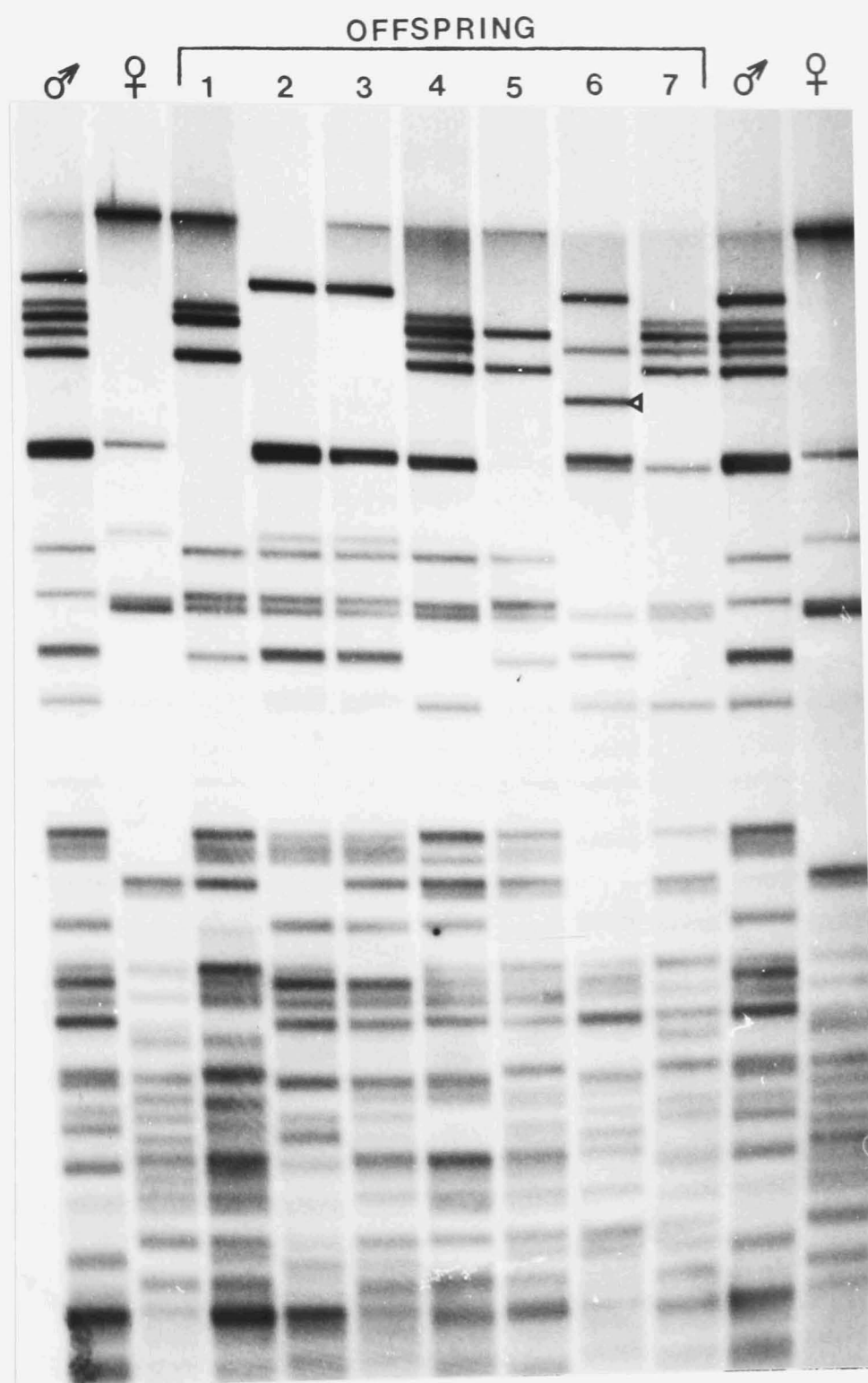
The origin of multiple mismatches was investigated using the band sharing coefficient

$$D = \frac{2N_{AB}}{(N_A + N_B)}$$

where N_A and N_B are the number of bands in two individuals and N_{AB} is the number

Figure 4.7

A newly arisen "mutation" in a House Sparrow family



A single novel band in offspring 6 cannot be assigned to either parent in an otherwise legitimate family. The enzyme genotype and pSPT 18.15 fingerprint pattern of this individual confirms its parentage. Therefore the band indicated by (◄) must be the result of a mutation.

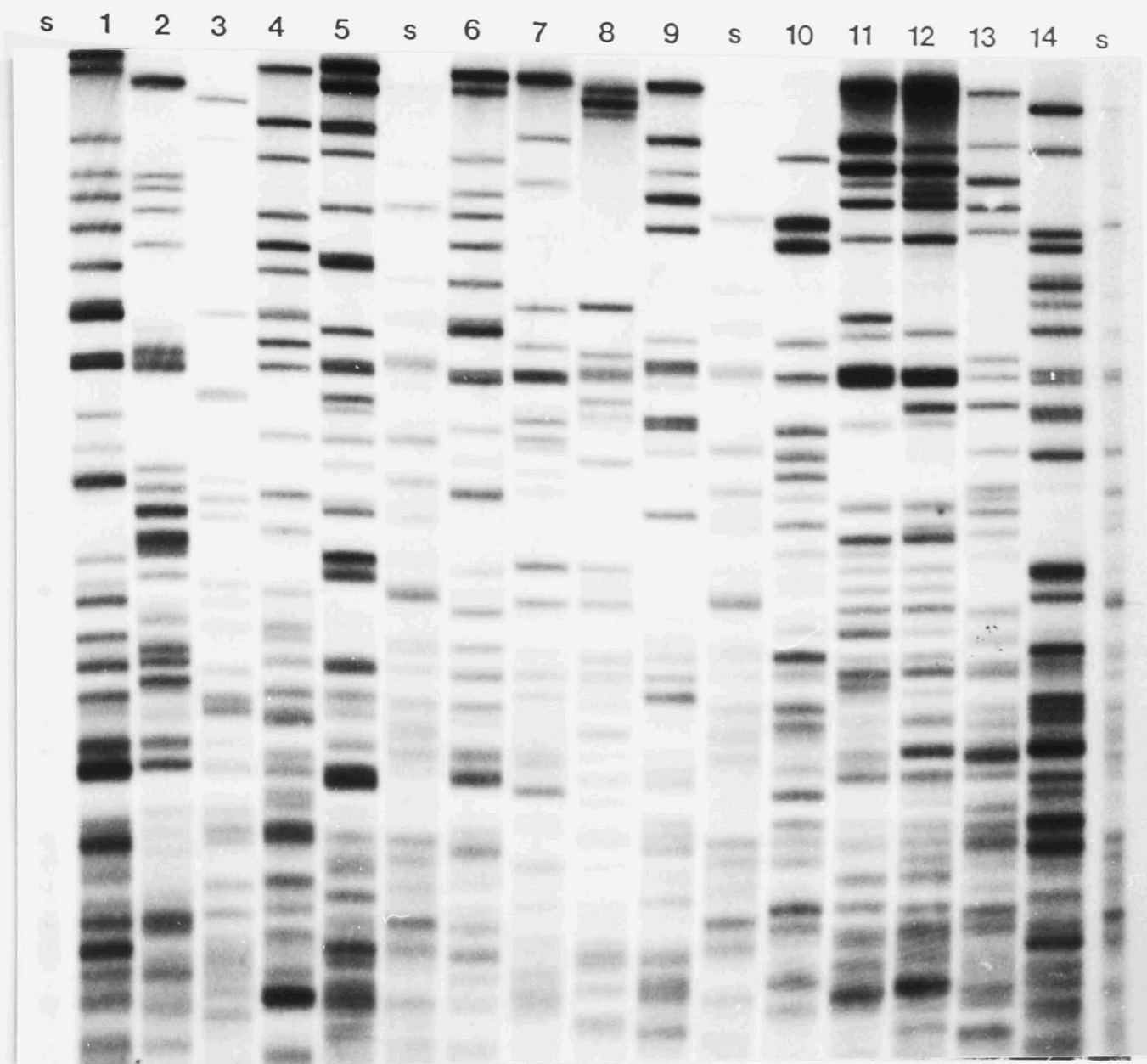
that they share (Lansman *et al.*, 1981, Appendix 4). Nine categories were compared based upon the sex and relationship of the individuals concerned. The background level of band-sharing between unrelated individuals was calculated from a comparison of all 36 mate pairs. These yielded a mean \bar{D} of 0.151 ± 0.019 similar to an analysis of unrelated males ($\bar{D} = 0.149 \pm 0.022$, $N = 16$) (Fig. 4.8). Fingerprints were compared only with immediately adjacent lanes to minimize misidentification of bands due to gel distortion.

The relationships within the 36 family groups were then investigated (Table 4.4, Fig. 4.9). The 24 multiple mismatches were assumed to have arisen through EPF and so were treated separately. The mutant individuals were pooled with the other legitimate, within-pair offspring (WPOs). Parents of both sexes were compared separately with EPOs and WPOs. The distributions of D values for female attendants were indistinguishable, implying that females are always related to their nestlings (i.e. no egg dumping). However, comparison of the two nestling classes with the male attendant produced virtually non-overlapping ranges. The \bar{D} of male/EPO comparisons (0.137 ± 0.022) is consistent with the value expected if males were unrelated to EPOs. This confirms that all cases of non-parentage resulted from EPCs between the attendant female and a non-mate. The comparison of male attendants with their true offspring yielded a significantly higher \bar{D} than that for nestlings and females. (This is a further reflection of the differences between male and female minisatellite phenotypes - see below.)

Comparisons were also made within sibships. As expected from the parentage analysis WPO/WPO comparisons produced a \bar{D} similar to parent/offspring values (i.e. 0.580 ± 0.009 cf 0.598 ± 0.009 for father/offspring and 0.541 ± 0.011 for mother/offspring). The coefficient of relatedness (r) corresponding to all these categories is 0.5, whilst EPOs and WPOs in the same brood will be half sibs ($r = 0.25$). The \bar{D} for this category is 0.341 ± 0.019 lying halfway between the values for

Figure 4.8

Fingerprints of 14 males which bred at Brackenhurst



The immense variability of House Sparrow fingerprints is evident from the rarity of shared bands in this sample. Individuals 12 and 11 which share many bands with each other are the only birds known to be related (father and son). The lanes marked S contain a standard to aid comparison across the gel.

Table 4.4

Summary of the similarity coefficient (D) analysis of the Brackenhurst family data

RELATIONSHIP		Mean D	±	S.E.	N
a	Male/Female	0.1511	±	0.0186	36
b	Male/Male	0.1492	±	0.0216	16
c	Male/WPO	0.5983	±	0.0085	173
d	Female/WPO	0.5362	±	0.0118	173
e	WPO/WPO	0.5802	±	0.0086	451
f	WPO/EPO	0.3411	±	0.0193	76
g	Male/EPO	0.1370	±	0.0218	24
h	Female/EPO	0.5775	±	0.0309	24
i	EPO/EPO	0.6507	±	0.0680	8

The basal level of band sharing between putative unrelated individuals was estimated from the mean similarity coefficient between (a) the two mated birds from 36 pairs and (b) 16 pairwise comparisons of adjacent fingerprint patterns derived from 18 males chosen at random from the aforementioned pairs (one coefficient was excluded as it involved a known father/son comparison). The similarity between first degree relatives was estimated from comparisons of (c) fathers and their offspring (d) mothers and their “legitimate” offspring (WPOs) and (e) “legitimate” full sibs. Females were equally similar to their EPOs (“illegitimate” offspring) and WPOs ((h) and (e) respectively) implying the attendant female was always the mother. Males compared with EPOs in their broods (g) gave similarity coefficients characteristic of unrelated individuals. EPOs and their attendants “legitimate” offspring (f) were clearly 2nd degree relatives from the intermediate value of their similarity coefficients (between first degree relatives $r = 0.5$, $\bar{D} = 0.58$ and unrelateds $r = 0$, $\bar{D} = 0.15$). The value for EPOs within broods (i) suggest that they are full sibs.

Figure 4.9

The distribution of band sharing coefficients between individuals of different relatedness

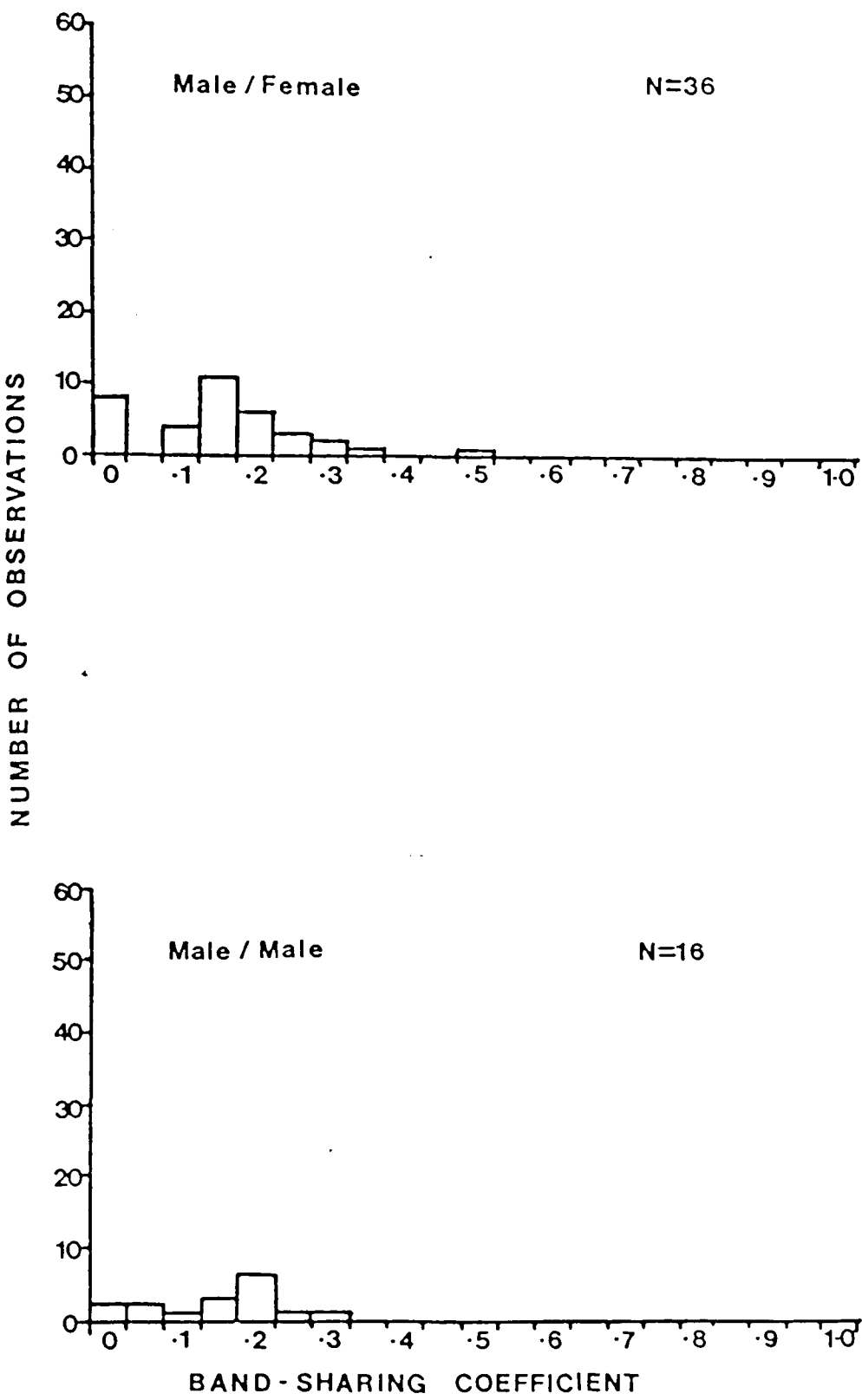


Figure 4.9 (cont)

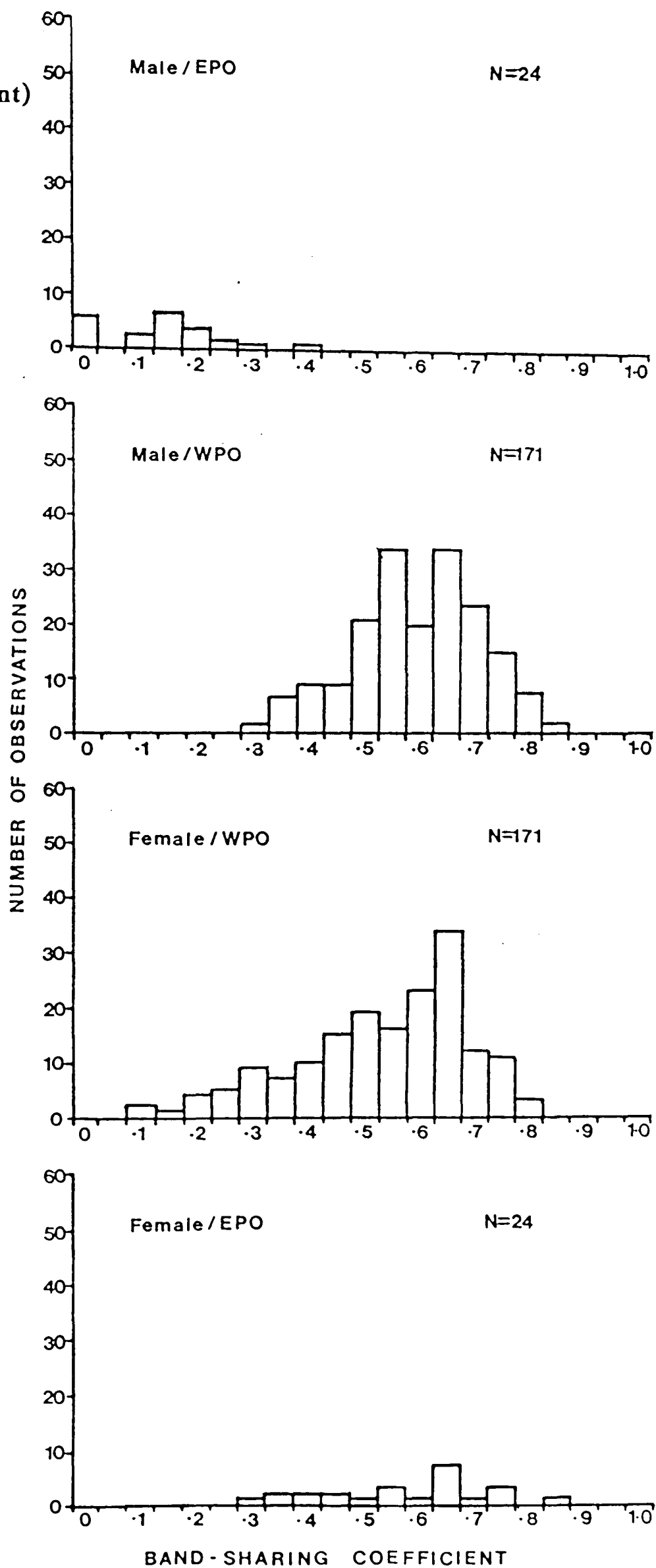
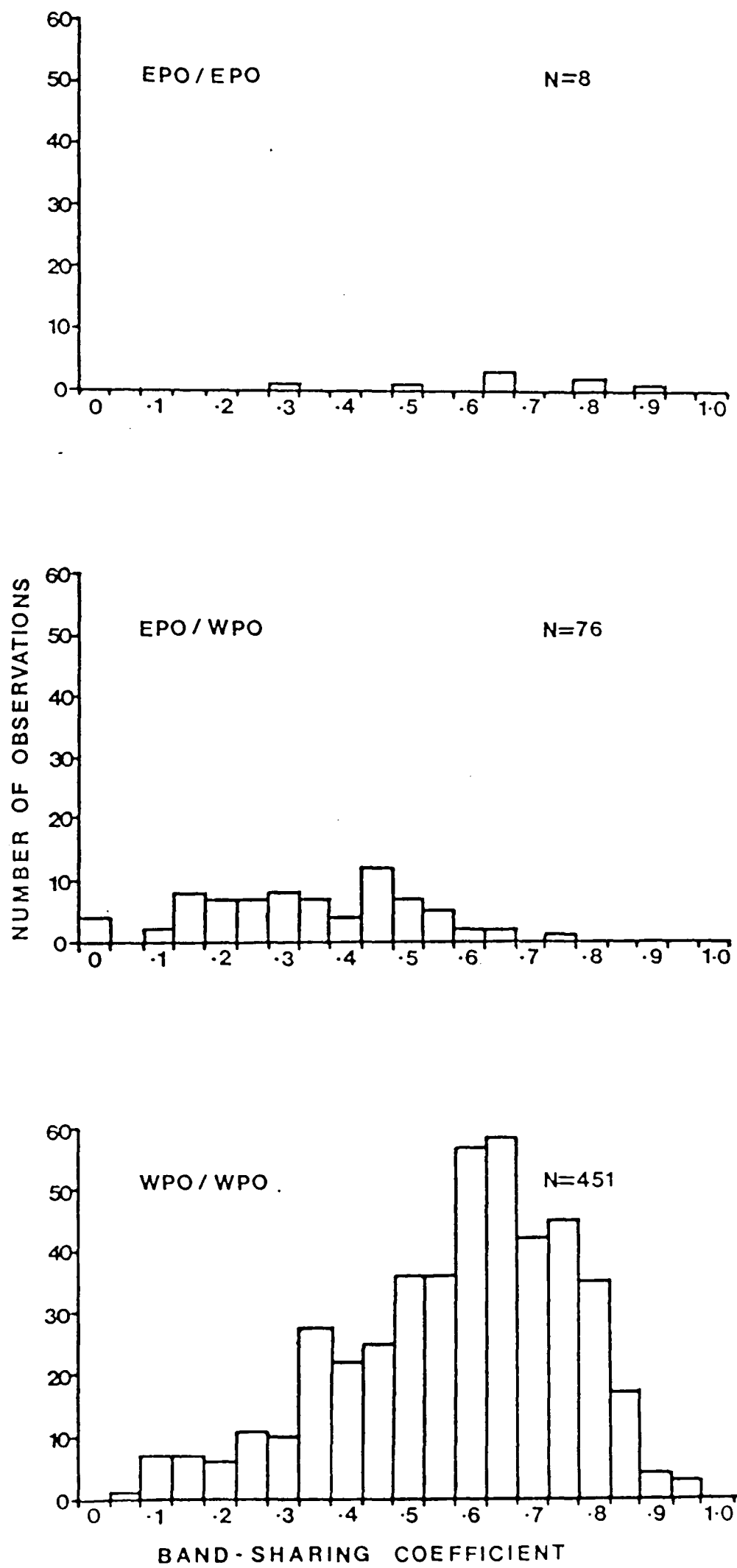


Figure 4.9 (cont)



unrelateds ($r = 0$) and first degree relatives ($r = 0.5$) but the distribution extensively overlaps both of the other ranges. The non-discrete distribution of second degree relatives (e.g. half sibs) prevents the use of similarity coefficients in discriminating between close relatives, whilst the limited overlap between the D values of attendant males with legitimate and EPO offspring shows they are useful in separating unrelateds from first degree relatives (96% discrimination being achieved with male attendants). Similar methods have been used to successfully discriminate between EPO and WPO Indigo Buntings (Westneat, 1990) and Purple Martins (*Progne subis*) (Morton *et al.*, 1990).

Finally EPOs within broods were compared in an attempt to determine the number of males contributing to a brood. The mean D value was characteristic of full sibs implying that in most cases a single extra-pair male was involved in each brood.

The House Sparrow fingerprints produced in this study have two unusual properties; males have on average three more bands, and two more allelic pairs, than females. Both are explicable by sex linkage on the Z chromosome. Recombination can occur in the homogametic (ZZ) males allowing dispersed loci to segregate independently, whereas all Z linked maternal bands will be inherited as a single haplotype. Hence maternal haplotypes should consist of more bands than paternal ones, although no evidence of this was found in the limited number of families examined.

The common occurrence of allelism in males and its rarity in females suggests that alleles at autosomal loci can vary greatly in size but they both rarely appear in the scorable high molecular weight region, whilst Z linked alleles are consistently large. Alternatively, if Z linked bands were frequently lost in the low molecular weight region, a large number of loci would have to be located on the Z chromosome to account for the difference in allelism between the sexes. This would, in turn, result in

much more frequent linkage between bands, particularly in females. Unfortunately, it was impossible to determine which bands were sex linked, as House Sparrows can only be sexed with confidence after the post-juvenile moult, six weeks after fledging.

The difference in band sharing coefficients between offspring and their mothers and fathers can be explained by sex linkage. If we assume the Z chromosome carries, on average, 3 bands (from the difference in the number of bands in males and females), we can see that sons inherit half of their bands from each parent whereas daughters can only inherit Z-linked bands from their father. Thus males make a larger contribution to the minisatellite complement of their offspring (Table 4.5).

Assuming the adults share no bands then the predicted \bar{D} values are 0.539 for males with their offspring and 0.469 for females. However, 15% of bands are shared by unrelated individuals and this must be taken into account. The proportion of bands shared by chance decreases whilst those inherited by direct descent increases in proportion to the coefficient of genetic relatedness. Therefore males and offspring will share 53.9% of bands by direct descent and approximately 6.9% $[(1-0.539) \times 0.15]$ by chance, i.e. 60.8% in total. Likewise the value for females is 54.9%. These are very close to the observed similarity coefficients, despite the numerous uncertainties involved, such as the assumption that band sharing does not vary greatly between autosomal and heterosomal loci. The mode of inheritance of these bands clearly warrants further investigation.

Sex linkage of minisatellite sequences that cross hybridize to the polycore probes has rarely been reported, even though other VNTRs are commonplace on human sex chromosomes. Jeffreys' found a single invariant X linked Hinf I fragment in mice which hybridized with 33.6 (Jeffreys, Wilson *et al.*, 1987). Burke's House Sparrow fingerprints revealed by 33.6 were dramatically different to those produced during this study (Burke and Bruford, 1987). He used Alu I which produced only 6 scorable

Table 4.5

Calculation of the expected mean similarity coefficient between parents and offspring

	PARENTAL SEX	
	MALE	FEMALE
Mean number of bands/adult	13.7	10.7
Mean number of autosomal bands	7.7	7.7
Mean number of Z-linked bands	6	3
Mean number of Z-linked bands transmitted to sons	3	3
Mean number of Z-linked bands transmitted to daughters	3	0
Mean number of autosomal bands transmitted to offspring	$\frac{7.7}{2} = 3.85$	$\frac{7.7}{2} = 3.85$
% of sons bands inherited directly from parent	$\frac{6.85}{13.7} = 50\%$	$\frac{6.85}{13.7} = 50\%$
Similarity coefficients with sons assuming no band sharing between the parents (D_o)	$\frac{2(6.85)}{(13.7 + 13.7)} = 0.5$	$\frac{2(6.85)}{(10.7 + 13.7)} = 0.578$
% of daughters bands inherited directly from parent	$\frac{6.85}{10.7} = 64\%$	$\frac{3.85}{10.7} = 36\%$
Similarity coefficient with daughters assuming no band sharing between the parents (D_o)	$\frac{2(6.85)}{(13.7 + 10.7)} = 0.578$	$\frac{2(3.85)}{(10.7 + 10.7)} = 0.360$
Mean D_o assuming 50:50 sex ratio	$\frac{(0.5 + 0.578)}{2} = 0.539$	$\frac{(0.578 + 0.360)}{2} = 0.469$
Adjusted \bar{D} assuming band sharing between unrelateds = 0.15 where $D_{adj} = D_o + 0.15(1 - D_o)$	0.608	0.549
Observed \bar{D}	0.598 ± 0.009	0.541 ± 0.011

bands larger than 2kb. Restriction sites for this enzyme must be very frequent in sparrow minisatellites homologous to 33.6, particularly those located on the Z chromosome. Conversely, Hae III digests produce very few bands which hybridize specifically to pSPT 18.15 (see Figs. 4.10 and 4.11) whereas Alu digests probed with 33.15 have a mean of 15.0 scorable bands.

With these data we are in a position to estimate the effectiveness of DNA fingerprinting as a demographic tool. The probability of unrelated individuals sharing a band is ~ 0.15 and the average individual has ~ 13 bands. From the segregation analysis of the nine largest families it was found that 23% of parent specific bands cosegregated perfectly with a previously scored band. If we assume that a similar proportion applies to shared bands, then an individual has 10 (i.e. $13 \times (1-0.23)$) unlinked bands including some which are allelic. 15% of the bands are shared by both parents, but approximately half of the remainder will be specific to each parent, i.e. ~ 4.2 bands. The probability of every paternal band being present in an unrelated male is $0.15^{4.2}$ which corresponds to an exclusion probability of 99.96%. If the true father was a close relative of the attendant male, the greater number of bands shared through common ancestry reduces the exclusion probability to 0.90 (i.e. $1-0.58^{4.2}$, where 0.58 is the mean proportion of bands shared by first degree relatives).

To allow for the possibility of a single mutation, the number of paternal specific bands can be reduced from 4.3 to 3.3. This still results in a satisfactory exclusion probability of 99.8% for cuckoldry by unrelated males, but only 83% for close relatives. Fingerprinting is clearly capable of detecting all EPOs produced through cuckoldry by unrelated males, but a significant proportion of EPFs by close relatives could go unnoticed. The latter event will occur very rarely due to the negligible proportion of the population made up by closely related males. The calculation given above assumed that equal numbers of bands were inherited from each parent, when in fact the average nestling shares more bands with its father, therefore the exclusion

probabilities are conservative estimates.

The fingerprinting survey revealed a further 23 EPOs in addition to the 28 detected by mismatches at the six enzyme loci. Starch gels detected 54.9% of these EPOs, fractionally more than the 54.7% predicted by the exclusion probability. The fit to expectation is remarkable considering the magnification of sampling error that is associated with low probabilities of detection.

All mismatches at the enzyme loci were confirmed as EPOs, with the exception of the two broods with aberrant “C” bands on general protein gels, and the only case of exclusion of an attendant female which occurred at PEPD3. The unusual appearance of the “C” bands suggested that these were artefacts caused by post-translational modification (see Chapter 3), whilst the zymogram of the mismatching nestling at PEPD3 appeared to be that of a perfectly normal C homozygote. This bird was probably heterozygous for a previously unsuspected null allele inherited from its mother.

No evidence of intra-specific brood parasitism was found even though egg dumping is common in some populations (D. Harper, pers. comm.). Every case of parental exclusion was the result of non-paternity. The 51 EPOs represent an EPF rate of 12.1%, a frequency that does not vary significantly between years (Table 3.12) despite changes in nest density and possibly sex ratio (see Table 2.6). 24% of the broods (N = 144) included some EPOs. Of these, the majority (N = 23) were of mixed parentage including some nestlings fathered by the attendant male. One brood of four which contained 2 EPOs may have been sired by three males from the dissimilarity of their paternal bands, although insufficient were scored to offer conclusive evidence. When more than one EPO occurred in the remaining broods, they often appear to share a higher proportion of the paternal component of their fingerprints than half sibs (see Table 4.4). This was also true of EPOs in consecutive broods raised by some pairs

(see Fig. 4.12). However, the exclusion probabilities associated with the technique as it was used during this survey are too low to assign parentage in the absence of the true father's fingerprint. Hence confirmation of shared paternity of EPOs will require a more sensitive approach using a different enzyme (e.g. Alu I) and both probes or a battery of single locus probes (Wong *et al.*, 1986, 1987).

EPF is clearly a significant phenomenon at the Brackenhurst population involving nearly a quarter of broods and 37% of the breeding males, and yet EPC has rarely been reported in studies of colour marked House Sparrow populations. Details of the reproductive behaviour of the Brackenhurst Sparrows are outlined in the following section.

Behavioural Observations

Observations of courtship and mating behaviour were made while identifying mate pairs for the analysis of family groups. Intensive observation of focal pairs to gain a detailed picture of mating behaviour was ruled out as being too time consuming, and so the data obtained^{are} somewhat anecdotal.

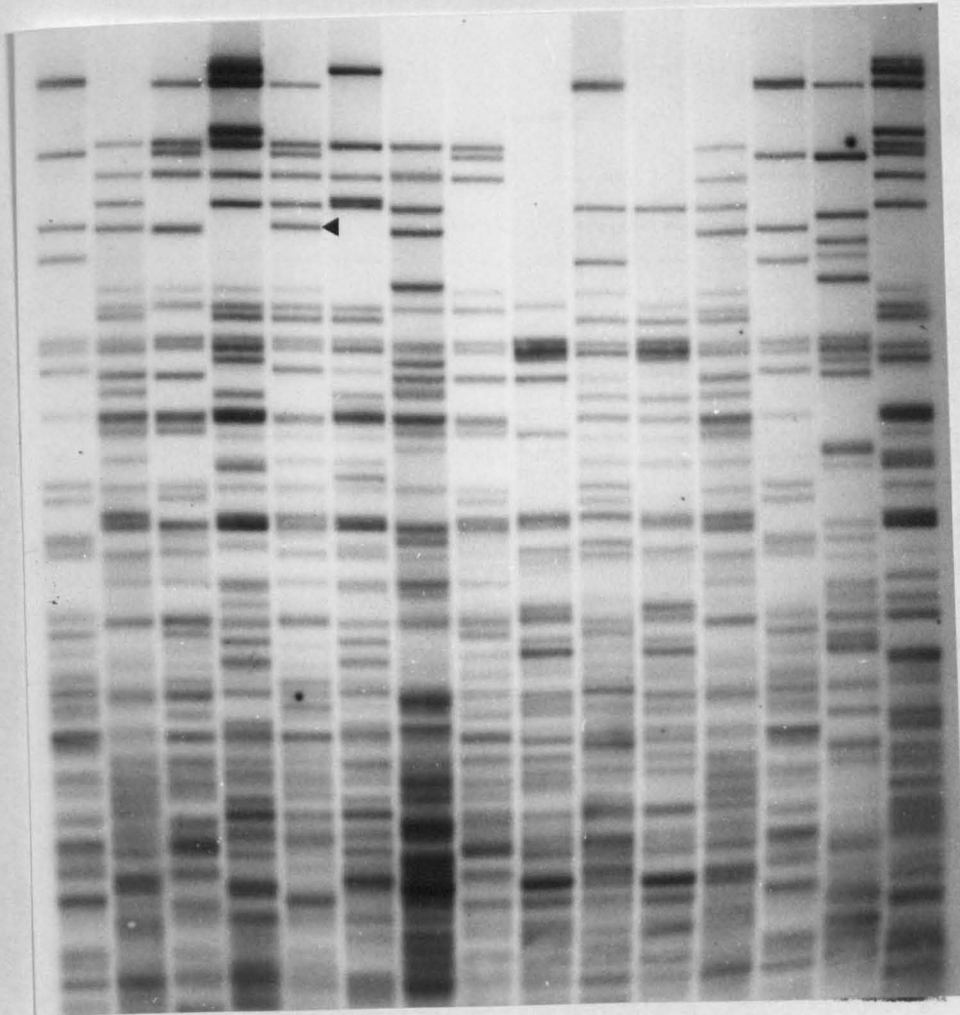
Whenever copulations were seen, an attempt was made to identify both birds. Most copulations occurred on or immediately adjacent to the nestbox. In 58 cases, both partners were clearly identifiable from their colour rings as the pair who attempted to breed in the box. The number of days between the observed copulations and the day on which the first egg was laid (day 0) is shown in Fig 4.13. A further 18 cases involved one ringed and one unmarked bird corresponding with those known to be using the nestbox. Since the identity of these birds is uncertain they are recorded separately. The observed copulations showed an almost identical temporal distribution with a published report of copulation behaviour (Møller, 1987a) and therefore probably

ORIGINAL THESIS CONTAINS
PLATES WITH CLEAR OVER-
LAYS.

THE FOLLOWING PLATE IS
SCANNED FIRST WITHOUT
THE OVERLAY, THEN WITH
THE OVERLAY IN PLACE

Figure 4.10

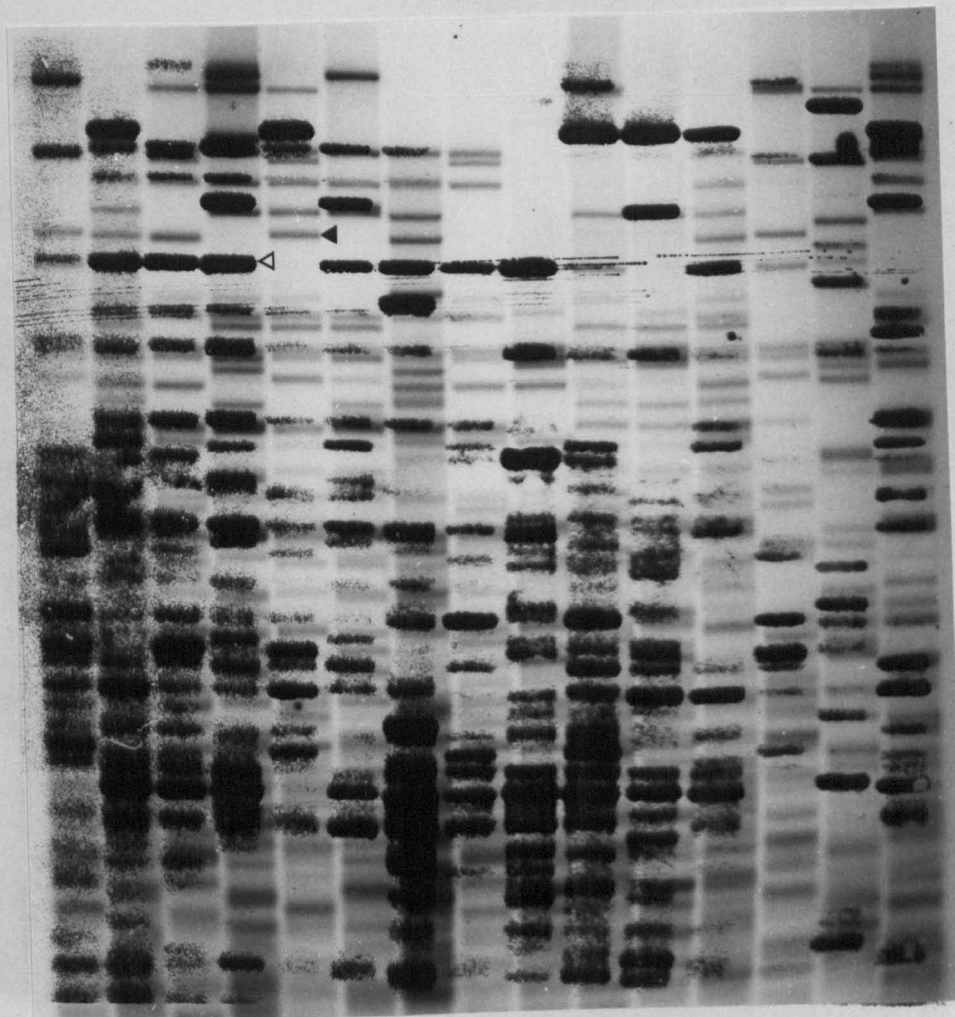
Hybridization to pSPT 18.15 at low stringency reveals cross homology to 19.6



The filter was initially hybridized to pSPT 19.6 at low stringency (1 X SSC, 1% SDS @ 65°C) to produce the above patterns, the filter was then stripped and rehybridized with pSPT 18.15 at the same stringency (see overlay). Some .6 specific bands are not revealed (e.g. ◀) whilst some .15 bands are detected (e.g. ◀). However, the majority of bands cross hybridize to both probes.

Figure 4.10

Hybridization to pSPT 18.15 at low stringency reveals cross homology to 19.6



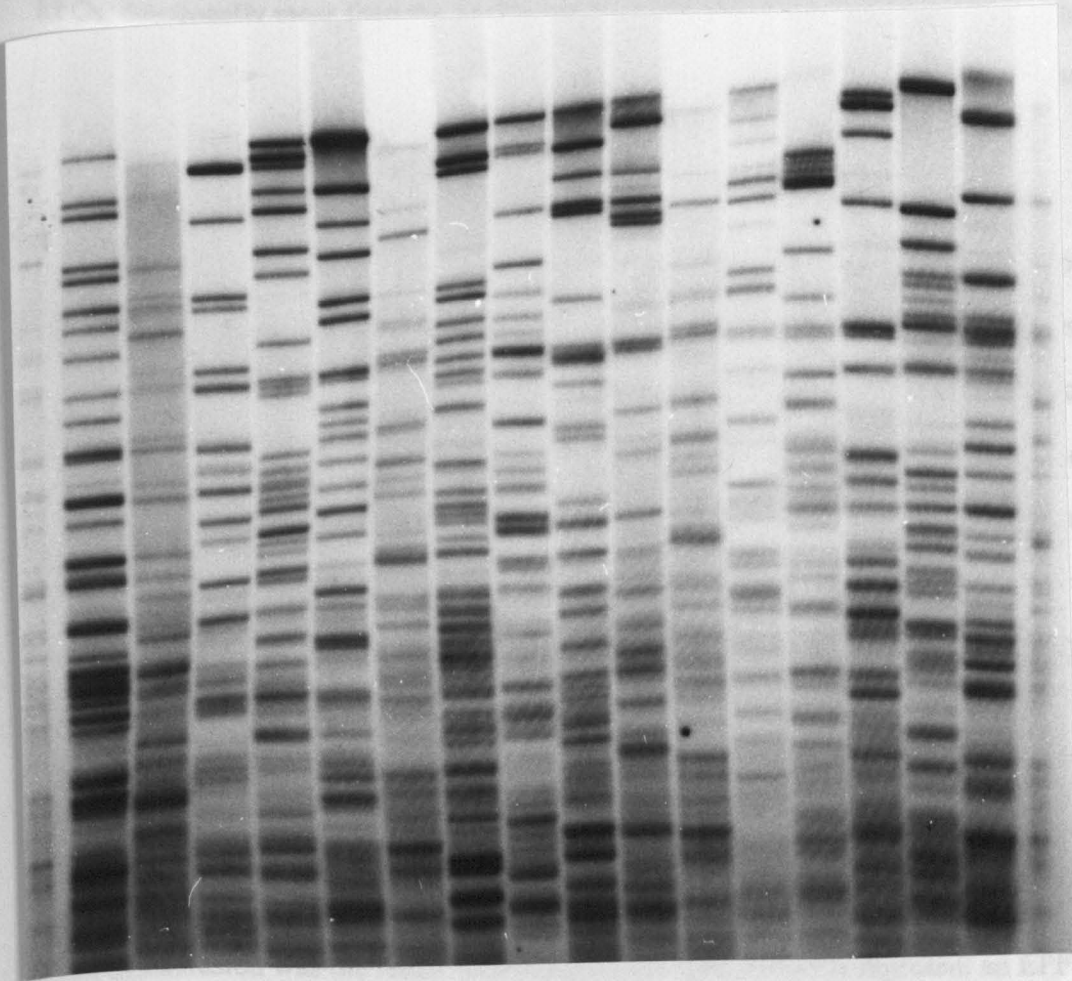
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Figure 4.11

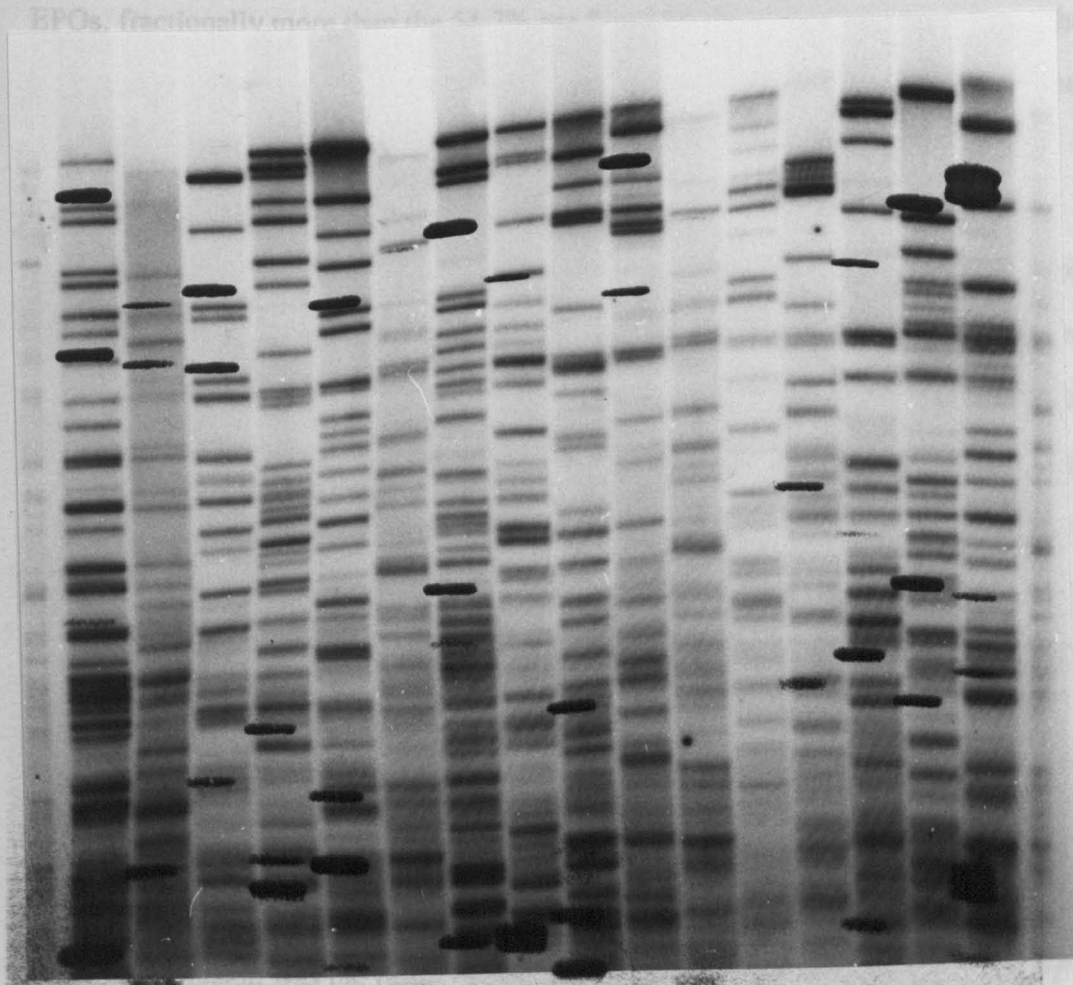
Hybridization to pSPT 18.15 at high stringency reveals probe specific bands



The filter was initially hybridized to pSPT 19.6 at low stringency (1 X SSC, 1% SDS @ 65°C) to produce the above pattern, the filter was then stripped and rehybridized with pSPT 18.15 at high stringency (0.5 x SSC, 1% SDS @ 65°C) to reveal the set of novel bands shown on the overlay.

Figure 4-11

Hybridization to pSPT 18.15 at high stringency reveals probe specific bands



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probabilities are conservative estimates.

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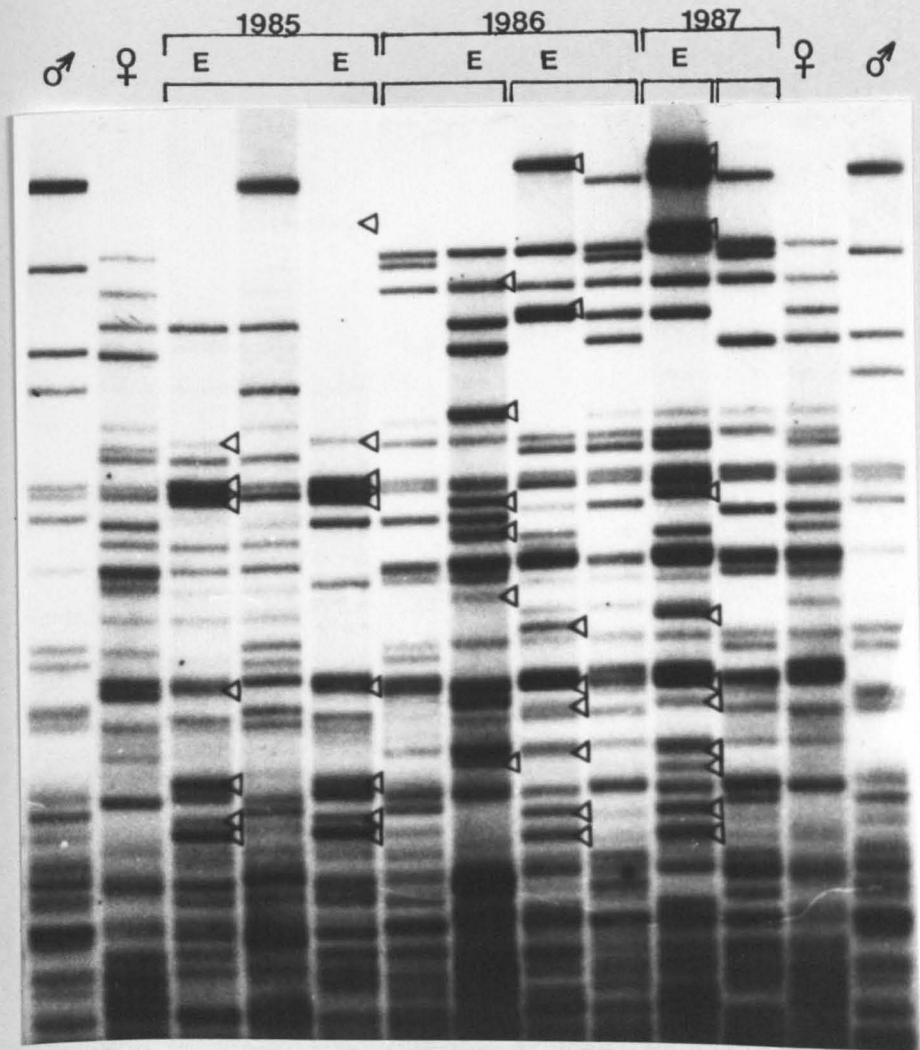
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Figure 4-12

An example of repeated cuckoldry



Five broods raised by a pair resident at Brackenhurst between 1985 and 1987. Five nestlings (E) clearly mismatch with the male but share many bands with their mother. The unassigned bands are probably derived from three other males. The two EPOs in the 1985 brood share many paternal bands, whilst the first EPO from 1986 shares none of its novel bands. The remaining EPOs from late 1986 and early 1987 again share many bands with each other.

This family provides evidence that females EPC with the same male on more than one occasion and that fertility may have an important role in determining the effectiveness of cuckoldry. Unhatched eggs were found in each brood except the last, which has no evidence of EPF.

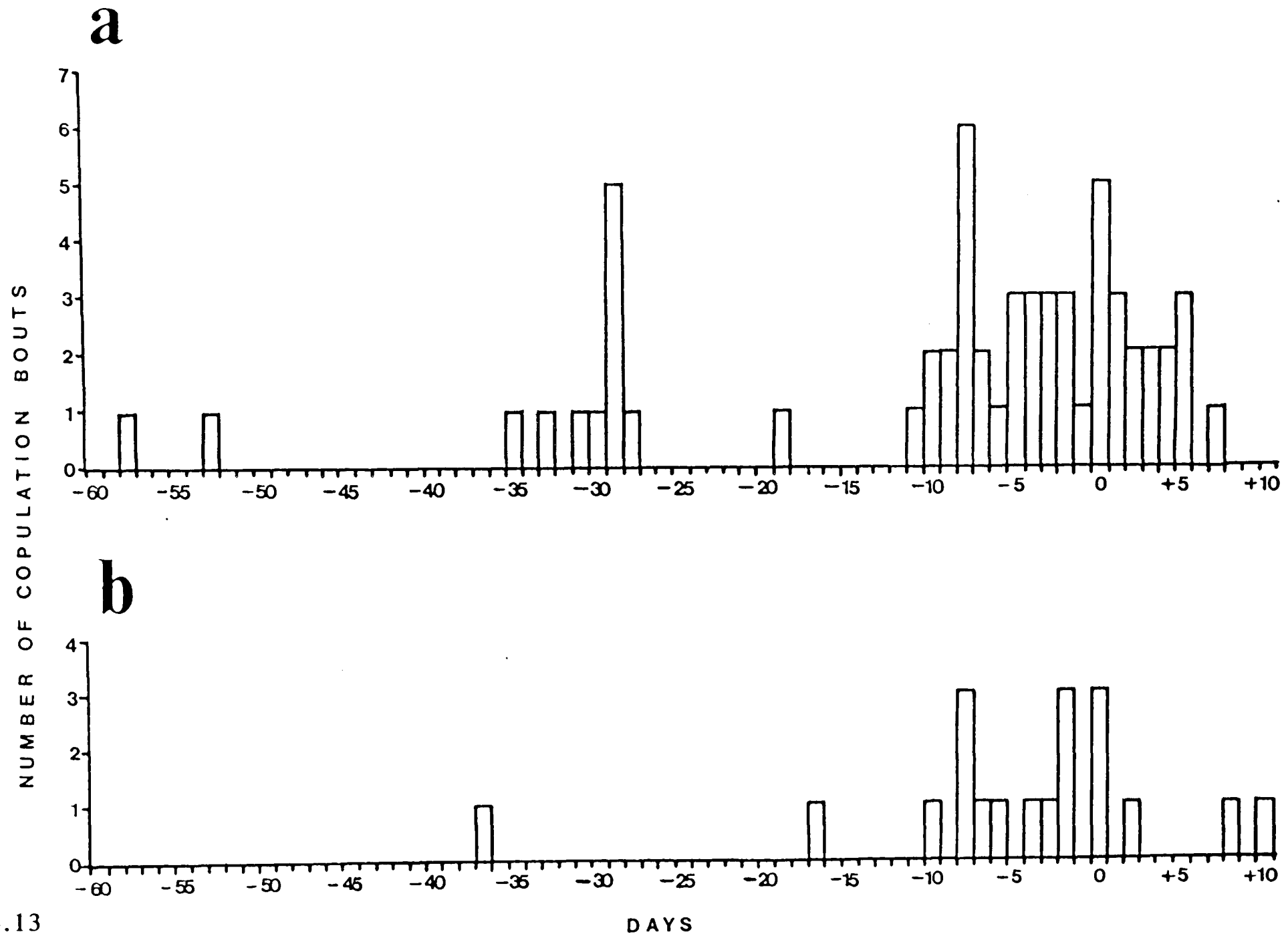


Figure 4.13

The temporal distribution of copulations relative to the date of clutch initiation (day 0). Figure (a) shows the timing of copulations between conclusively identified adults (b) represents the distribution of copulations including a single unrung adult corresponding with the known nest attendants.

represent a random sample of matings.

Copulations were observed as early as the 3rd March, 53 days before the first egg was laid. A breeding attempt at this time of year would certainly fail through lack of food for the nestlings. Presumably very early copulations are part of pair bond formation or maintenance. Sexual activity was sporadic until about 10 days before laying commenced, when it increased and remained high until the last egg was laid, after which copulation declined abruptly.

EPCs were rarely observed, but may have been involved in the following cases. Firstly, a copulation was observed in which only part of the colour code of each participant was seen. The colour rings were consistent with the male occupying the nestbox but the colour combination of the female did not correspond with any female using the nestboxes in that year. Another female copulated with two males in an 8 day period. Unfortunately, she occupied a concealed natural nest making it impossible to identify her mate, so the possibility of a mate change cannot be excluded. Finally, a female was observed copulating with the occupant of a neighbouring box. Her mate returned, chased the cuckolder away and immediately copulated with her. Unlike virtually all observed within-pair copulations, the female did not solicit her mate's attention by crouching, shivering her wings and chirruping quietly, but remained totally passive. The copulation occurred on day -2 and the subsequent brood included an EPO and two legitimate young. The EPOs enzyme genotype is compatible with the neighbours but paternity has not yet been confirmed by fingerprinting. In all three cases the female cooperated in the EPC attempt but the preceding behaviour was not seen. All other observations of actual copulations were incomplete but none provided any evidence of EPC.

Males frequently displayed to females, in a characteristic manner with wings held out, the head raised vertically to display the bib, and the tail raised and spread. On all

occasions when this behaviour was seen, the female was not the male's mate or his mate was unknown. The display is apparently given to attract a mate or to solicit an EPC but was always unsuccessful; females either ignore or attack displaying males before flying off. This often initiated the behaviour which Summers-Smith called the communal display but which is more commonly known as a "sparrow wedding" (Summers-Smith, 1955; Møller, 1987a).

Males follow the fleeing female, calling loudly, which attracts other males in the area to the chase. If the female lands, males surround her displaying frantically, often oblivious to the approach of observers, cars or predators, such as cats. Females attempt to remain facing the males and occasionally lunge at them. Males which succeed in getting behind the female will mount and attempt a forced copulation (an FEPC). However, this is rarely successful due to the lack of an intromittent organ and the female's vigorous resistance. Usually females attempt to escape into cover such as a dense bush. Failing this, they back into a corner or even into the tread of a tractor tyre to protect their cloaca. Females caught in the open courtyard simply squatted against the ground. FEPC attempts during communal displays are aimed specifically at fertile females and are particularly frequent during the first brood (Møller, 1990).

Møller (1987a) has recorded one further aspect of communal displays which was not seen at Brackenhurst. Males at his colony pecked at the females cloaca presumably to force them to eject the contents of their sperm storage glands. Similar behaviour occurs in the Dunnock where males of polyandrous trios do this to remove sperm introduced by inseminations from the other male (Davies, 1983).

Communal displays end when the males disperse. On one occasion the mate of a female involved in a display arrived and attacked the departing males. The female then left cover and started to solicit to her mate who copulated immediately. (A similar event is reported in Summers-Smith 1955.) Communal displays are the most frequently

observed situations in which EPCs are attempted. However, they are unlikely to contribute many EPFs as cloacal contact is very rarely achieved. All within pair copulations were initiated by the females solicitation display during which the tail is raised to expose the cloaca. Only once was a female seen soliciting a male other than her own mate. However, the outcome was not observed as the birds were disturbed by human activity. Solicited EPCs may be much more common than observations suggest if they are carried out surreptitiously. Copulations have frequently been seen and heard in dense hedgerows, many yards from the nearest nest site even though all identified within pair copulations occurred within a couple of metres of the participants nest site.

Intensive observation of several small House Sparrow populations has revealed that females prefer to mate with males holding nest box sites. Failing this they will pair with males occupying inferior, predation prone nest sites in hedgerows. The preferred sites are held by males with large bibs. These birds are more likely to find a mate, be polygynous and provide a high level of parental care (Møller, 1989a) They are also more likely to take part in FEPCs and solicited EPCs particularly with females from poorer sites (D. Harper, pers. comm. and Møller, 1990). These observations suggest that some females may be frustrated in their choice of mate.

At Brackenhurst, females mated polygynously in each year from 1985. Polygyny may have occurred in previous years but the lower percentage of ringed males lessened the chances of detecting it. Whether a lack of males or nest sites was responsible is hard to determine. Papers cited by Summers-Smith (1963) suggest that some populations include unpaired individuals that are available to rapidly replace birds of either sex when one member of a pair is removed, although these birds may have been prevented from breeding by a lack of nest sites. This situation does not apply at Brackenhurst where at least 50% of boxes are unoccupied in each year, despite being successfully used in previous years. Regardless of the cause, polygyny is rarely a successful strategy at Brackenhurst.

Bigamous males help to feed the first brood to hatch, aiding the secondary female only if the primary brood fails. In most years, two adults were required to successfully raise young to fledging, and so most secondary broods failed or fledged only a single individual, although both females successfully raised all hatchlings in a polygynous trio in 1989 when food was exceptionally plentiful (see Fig. 4.14). Polygynous males usually occupy two adjacent boxes, although one trio used nests separated by 20' and an intervening box. Males apparently made no attempt to deceive their mates with respect to their "marital status", unlike male Pied Flycatchers who, having acquired a mate and fertilized the eggs, then attract a second female to another territory some distance from the first. Once the secondary female is incubating the male abandons her to help the primary female.

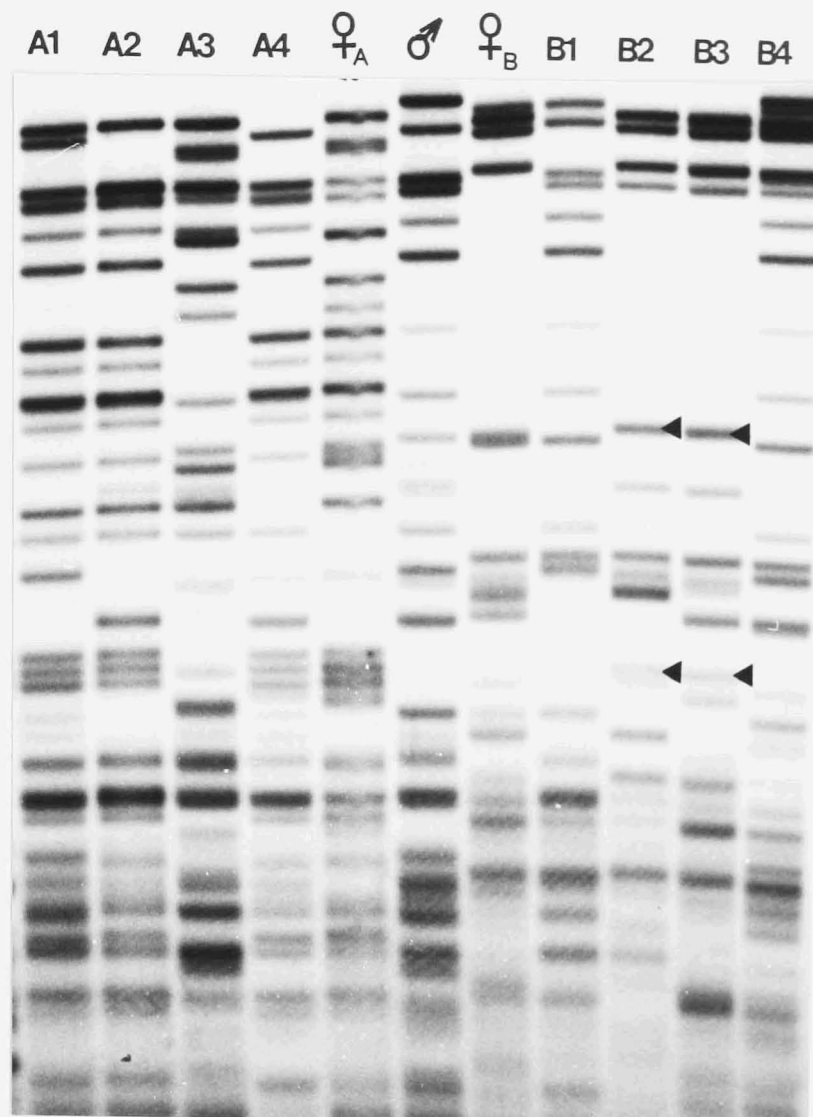
The frequent occurrence of polygyny, Harper's observations of EPCs with particular males, and the fingerprint evidence which suggests that some females EPC with the same male on several occasions, raises the possibility that females perceive a difference in quality between males and choose to mate with the preferred males while strongly resisting attempts by others (Møller, 1988a, 1990). Likewise female swallows prefer long-tailed males as mates and are more likely to resist EPC attempts by short-tailed males (Møller, 1988b). What traits might be involved will be investigated in future sections, with particular attention being paid to the role of bib size and tail length (see Chapter 5).

The Distribution of EPOs

A striking feature of the observational data is the lack of evidence for EPCs. A comparison of the observed EPC rate of 1 in 59 with an EPF rate of 51 in 420 reveals an excess of EPFs ($\chi^2 = 5.06$, $P < 0.05$). Westneat (1987a and b) found a similar

Figure 4.14

A successful case of polygyny



One of the few successful cases of polygyny was confirmed by DNA fingerprinting the three broods raised by the male and his mates. Female A raised a single brood of four all of which were fathered by the male. Meanwhile female B raised a brood of three (B2-4) which contained two EPOs (B2 & 3). Unusually the male fed both broods. A second brood raised by female B failed but a fingerprint derived from the corpse of the last nestling (B1) confirms the males paternity.

excess of EPFs in a population of Indigo Buntings despite a more systematic approach involving following individual females for periods of 90 minutes, although they were occasionally lost from view for several minutes. EPCs accounted for 12.8% of 413 observed copulations but due to the females resistance only 3.3% of EPC attempts achieved cloacal contact and yet the EPF rate estimated by starch gel electrophoresis exceeded 30%. A deficiency of EPCs relative to EPFs has also been found in the Swallow (Møller, 1985 and 1987c).

Discrepancies of this nature may arise purely by chance given the limited number of copulations observed which, in both studies, were a tiny proportion of matings involving each female. A bias towards WPCs will exist because they typically involve a period of display and solicitation followed by several mountings whereas forced EPCs are typically very brief and solicited EPCs, if they occur, are likely to be secretive and concealed from the mate. Polyandrous female Dunnocks go to great lengths to escape from the α male so that they can mate with the β male (Davies, 1983). By so doing they encourage both males to provide parental care to broods in which they may have an investment. The amount of care given by the male is strongly correlated with the number of copulations and the proportion of the brood sired and therefore reflects the males confidence of paternity (Burke *et al.*, 1989).

Timing is another important factor in the success of avian copulations. Although sperm storage is highly developed, with records of domesticated birds laying fertile eggs more than 50 days after the last insemination, such prolonged storage is of little relevance to wild birds because of the decline in fertility, hatchability and survival of young. However, a short period of storage may increase fertility with maximum hatching success from inseminations 2 to 3 days before the egg is laid (Sturkie, 1976).

Ovulation occurs early in the morning shortly after the previous egg is laid. Then follows a short "fertilization window" of 15 to 30 minutes before the first layer of

albumen is laid down in the infundibulum preventing sperm from reaching the ovum (Sturkie, 1976). Either stored sperm or a fresh insemination can fertilize the egg, so there is a peak of sexual activity early in the morning. Sperm storage probably evolved to ensure that some would be available if the female was unable to mate during this short period, a common occurrence in, for example, seabirds that forage away from the colony.

The effectiveness of storage increases the probability that sperm from different males will compete to fertilize the egg. The importance of timing in sperm competition has been emphasized by experiments using Zebra Finches with heritable plumage traits (Birkhead *et al.*, 1988 and 1989). Females separated from their mate, and exposed to a single EPC from a male with a different plumage trait produced broods containing 54% EPOs, despite having received an average of nine within-pair copulations (WPCs) before the single EPC. Correcting for failure to transfer sperm during some EPC attempts increases the estimate of sperm precedence for the last insemination to 84%. Because a single insemination can fertilize an entire clutch and the last male to copulate will sire most of the young, males will compete to be the last to inseminate a female, and will therefore mate many more times than is necessary to fertilize the eggs (Birkhead *et al.*, 1987).

To ensure paternity of a brood a male can either guard his mate against EPC attempts from other males, or copulate frequently in an attempt to dilute or displace inseminations from others (Birkhead, 1987). The former strategy is widely used by territorial monogamous birds who can exclude intruding males from access to the territory and the fertile female. However, mate-guarding is usually unproductive in colonies where the high density of males leads to frequent EPC attempts. House Sparrows copulate as often as 40 times a day during the females fertile period (from day -10 to the day the penultimate egg is laid) (Birkhead *et al.*, 1987). The frequency of within pair matings will ensure that the majority of nestlings are fathered by the

attendant male and the occurrence of several EPOs within a brood will be rare. Therefore the presence of several broods from which the attendant male is totally excluded from parentage is very surprising.

Evidence for the correct identification of the male at the 12 broods where the attendant made no genetic contribution is given in Table 4.6. Only three males never raised a legitimate offspring. These individuals might have been excluded because of errors in the application of colour rings or mislabelling of the blood sample. Great care was taken to avoid mistakes and no evidence of their occurrence exists. The correct identity of the blood sample of the BH20 male is proven by the perfect correspondence of this sample with the fingerprints of his parents. The correct labelling of the samples belonging to the two other males (from BH15 and BH33) cannot be confirmed but genotypes of the other birds sampled on the same day also excluded them from parentage.

An alternative explanation of totally excluded broods is adoption of the brood by a male following the death or desertion of the female's mate. Artificially widowed Savannah Sparrows (*Passerculus sandwichensis*) can acquire mates which help to rear broods they cannot have fathered (Weatherhead and Robertson, 1980), while experimentally widowed or secondary female Pied Flycatchers will solicit copulations from other males even though the clutch is complete, a behaviour not seen in stable monogamous pairs (Gjershaug *et al.*, 1989). Some new males are apparently deceived over their investment in the brood into providing parental care, just as polyandrous Dunnocks will gain aid from both males to raise a single nestling (Burke *et al.*, 1989).

A possible case of mate deception involved a pair that raised two broods, the first containing a single mismatch, the second being entirely fathered by the male thought to be responsible for the EPO in the first brood (see BH28 in Table 4.6). While the second brood was being laid the only male attending the nest was the father of the

Table 4.6

Evidence for the correct identification of the attendant male at totally excluded broods

SITE	DATE OF BLEEDING	MALE IDENTIFIED WHILST ATTENDING					
		Earlier Brood		Mismatching Brood during			Subsequent Brood
		At same box	With same female	Fertile Period	Incubation	Feeding	With same female
BG05	060686	None	None			✓	✓
BH21	250868	✓	✓			Not seen	✓
BH28	130886	x	✓	Different male seen	✓	✓	None
BH20	010687	None	None			✓	✓
BH15	010787	None	None			✓	✓
BH21	220787	✓	✓		✓	✓	None
BH08	240787	✓	✓			✓	None
BH20	030887	✓	✓			✓	None
BH33	170887	None	None			✓	None
BH36	230588	✓	✓	✓	✓	Not seen	Mismatch brood polygynous
BH51	250588	✓	x			✓	✓
BG42	290788	✓	✓		✓	✓	Different female

EPOs who was seen lining the nest with feathers. This male was not seen again after the last egg was laid until the following year. However, the initial mate was seen at the nest three days after the last egg was laid, copulating with the female and subsequently fed the nestlings. Copulation after completion of the clutch is rare in House Sparrows (<5% of observed matings), and in no other case were two males seen to contribute to the care of the brood.

Mate replacement is unlikely to be a significant cause of paternity exclusions as more than half of the cases of total exclusions occurred in broods raised by established pairs in the nest used for their previous breeding attempt. Having rejected the possibility of frequent field error and mate replacement, only EPCs remain as a source of EPOs.

Since the majority of EPFs arise through EPCs an excess is expected in the first broods corresponding with the maximum frequency of communal displays and the forced EPC attempts which accompany them (Summers-Smith, 1955 and Møller, 1987a). The rapid decline in the number of displays as males become involved with raising their own nestlings should be tracked by a decline in EPFs, but an analysis of variance shows that EPOs are uniformly distributed throughout the breeding season. The frequency of communal displays presumably varied between years as well but as previously mentioned the EPF rate was effectively constant between years. Thus there is little evidence that FEPCs result in EPF.

The density of breeding pairs is also likely to influence EPCs but is virtually impossible to quantify. All the boxes in the farm complex are within foraging distance, as are an unknown number of natural nests. Breeding attempts are not synchronized, so the number of birds at different stages in the nesting cycle will vary.

Other factors that might influence the distribution of EPOs are the age of the

attendant adults and their previous breeding experience. Age had no detectable effect, although the only mixed brood containing three EPOs was the last brood of the oldest male in the colony who was at least six years of age.

Broods containing EPOs were equally likely to be raised by newly formed or established pairs and the occurrence of EPF had no effect on subsequent breeding attempts or the likelihood of a pair “divorcing”, i.e. each bird breeding subsequently with a new partner. Nine divorces were recorded, of which three followed broods containing EPOs.

Despite the lack of correlation of EPF rate with these factors, EPOs were not randomly distributed. There was no difference in initial clutch size between legitimate broods and those containing EPOs, and the brood size at fledging was not significantly different. But a significant bias exists in the total number of EPOs and legitimate offspring from broods varying in fledging success which was largely due to an excess of EPOs in broods with a sole survivor (Table 4.7, $\chi^2 = 19.3$, $df = 4$, $P < 0.001$).

In order to identify the reason for the excess of EPOs in small broods, the proportion of eggs hatching, of nestlings surviving to fledge and of nestlings mismatching with the attendant male, were calculated for each brood. Variation in the proportion of EPOs was almost entirely due to poor hatching success ($P < 0.001$). Furthermore, testing the contingency table of hatching success against proportion mismatching revealed a significant linear trend of increasing probability of EPF with declining hatching success ($P < 0.01$, $df = 1$) (Table 4.8) (McCullagh, 1980).

Broods were thus classified into four categories:

- A) No loss at any stage.
- B) 100% hatching success but some nestling mortality.
- C) <100% hatching success but no nestling mortality.

Table 4.7

(a) The fledging success of EPO containing broods

Number of fledglings	Number of broods		
	without EPOs	with EPOs	
1	10	7	17
2	22	8	30
3	40	12	52
4	33	8	41
5	4	0	4
	109	35	144

$$\chi^2_4 = 4.56, \text{ N.S.}$$

(b) The distribution of EPOs between broods varying in number of fledglings

Number of fledglings	Number of		
	legitimate offspring	EPOs	
1	10	7	17
2	50	10	60
3	136	20	156
4	150	14	164
5	20	0	20
	366	51	417

$$\chi^2_4 = 19.29, P < 0.001$$

N.B. Three nestlings starved after the date of ringing and thus failed to fledge. All were legitimate and from separate broods (the eventual number fledged was 2, 3 and 3)

Table 4.8

Classification of fully sampled broods on the basis of percentage nestlings sired through EPCs and proportion of eggs hatching. Blood sampling occurred after most nestling mortality

% Hatch	% Mismatch				
	0	25-33	50-67	75-100	
25-33	2	0	0	3	5
50	2	0	2	0	4
60-67	4	0	1	1	6
75	12	1	1	0	14
80-83	7	3	1	2	13
100	82	6	7	5	100
	109	10	12	13	144

D) Losses occurred both pre- and post-hatch.

The data are presented in Table 4.9 along with the χ^2 values testing the effect of loss at each stage on the proportion of EPOs. The EPF rate in broods with no unhatched eggs was 10.7%, significantly less than the 19.6% recorded in broods with some failed eggs ($P < 0.01$). There was no evidence of differential mortality of EPOs ($P > 0.5$), thus eggs would appear to be almost twice as likely to have been fertilized by an EPC when others in the same brood fail to hatch.

During the course of the fingerprinting survey, unhatched eggs were left in the nest until disposed of by the birds, so the stage at which the eggs failed is unknown. Seel (1968b) examined 2930 eggs that were incubated for the normal period. 12% did not hatch, of which a third showed signs of embryonic development, and the remainder were apparently infertile. Eggs examined at Brackenhurst before 1985 and during 1989 support these findings.

A significant correlation between EPFs and embryo mortality is unlikely. Incubation is the responsibility of the female, the male lacks a brood patch and merely covers the eggs to prevent chilling during the females short feeding bouts. Males with a low confidence of paternity because their mates had been involved in frequent EPCs might reduce their investment in the brood by helping less during incubation. However, the evidence of a significant linear trend connecting EPF rate and hatching success would imply that males were reducing their investment in relation to the number of eggs that they had fertilized despite the fact that the entire clutch would be affected equally by chilling and that excluded males were subsequently observed providing parental care to the nestlings as frequently as fathers of legitimate broods.

A possible association between infertility and success of EPCs is of particular interest as insurance against mate infertility is one of the benefits that might be gained

Table 4.9a

The distribution of EPO containing broods with respect to hatching and fledging success

Category	Hatching Success (%)	Post-Hatch Survival (%)	No. of Broods	Number of		% EPOs
				EPOs	WPOs	
A	100	100	38	11	135	7.5
B	100	<100	63	19	145	13.1
C	<100	100	27	13	58	18.3
D	<100	<100	16	8	28	22.2

Table 4.9b

Breakdown of determinants of EPO distribtuion

Categories	χ^2	df	P
A versus B	1.45	1	N.S.
C versus D	0.23	1	N.S.
A+B versus C+D	7.33	1	<0.01
A v B v C v D	8.86	3	<0.05

by a female through indulging in EPCs (McKinney *et al.*, 1984). If the female's mate is infertile or produces insufficient sperm to ensure fertilization in the limited time available, the female risks a considerable investment in time and resources which can be at least partially protected by insemination by more than one male.

Males will undoubtedly vary in fertility both between individuals and with time. To my knowledge there ^{are} _^ no data on sperm count variation in passerine birds, but in domesticated species counts vary with testes size (Møller and Erritzøe, 1988) and the number of sperm per ejaculate decline with multiple matings (Møller, 1990). The high copulation rate within House Sparrow pairs may deplete sperm reserves faster than spermatogenesis can replace them. Thus, there may be a progressive decline in the fertility of ejaculates during the fifteen days of frequent mating before the last egg is laid. Males whose mates are not fertile may accrue larger reserves and produce more potent ejaculates, hence the stage of the extra-pair males breeding cycle may be of great importance in sperm competition. In a competitive situation the male providing the most sperm has the greatest probability of fertilizing the egg (Martin and Dzuik, 1977 and Martin *et al.*, 1974).

Some mixing of sperm from separate inseminations does occur as shown by the Zebra Finch study cited above. The nature and location of the mixing of ejaculates within the female reproductive tract is unknown but is likely to involve either the displacement of the previous ejaculate or their sequential layering in the sperm storage gland.

If separation of ejaculates is almost complete, then one consisting almost entirely of seminal fluid or in which the majority of sperm are inviable, will be almost as effective as a normal one in preventing the previous insemination from achieving fertilization. However, if partial mixing occurs in the storage gland then a single potent ejaculate from a non mate may contribute enough sperm to fertilize a proportion of the

eggs even after repeated dilution with seminal fluid deficient in sperm. A mate of normal fertility will introduce sufficient sperm through frequent WPC to outcompete those from other males and EPFs will arise mainly when the last insemination was extra-pair. The observed EPF rate of 9.0% in fully fertile broods suggests that less than 10% of successful copulations are extra-pair during the immediate laying period and overall may be much rarer.

Summary

DNA fingerprint patterns were produced by probing Hae III restricted House Sparrow DNA with radiolabelled RNA transcripts of the insert of pSPT 19.6 which is the subcloned polycore minisatellite fragment from the Jeffrey's clone 33.6. The patterns revealed by autoradiography were derived from many highly heterozygous loci, each individual possessing on average 8 scorable independently segregating fragments. Males possessed more bands within the size range 5 - 30kb of which a significantly larger proportion were allelic than in females. These observations are consistent with Z chromosome linkage of a number of bands.

An examination of 420 nestlings from 144 broods at which both attendant adults were identified revealed three which possessed a single "mutant" band and 51 with multiple mismatching fragments. The high proportion of bands shared with the attendant female but lack of paternal specific bands indicated an EPF rate of 12.1% with an exclusion probability of 99.96% for non paternity due to EPF by an unrelated male. No evidence of egg dumping was found.

Females were frequently exposed to forced EPC attempts by males during communal displays. These were vigorously resisted and very rarely could have led to sperm transfer. Solicited and unresisted EPCs also occurred but were seldom observed. Very frequent within-pair copulations peaking at the start of egg laying were a response to the threat posed to the attendant males paternity of the brood by EPCs. EPFs were significantly more common than the observed EPC rate would suggest.

The EPF rate did not vary between years or during the course of the season providing strong evidence that most EPFs are not the result of FEPCs during communal displays which are significantly more frequent during the first brood. The distribution of EPOs between broods was unrelated to the age or breeding experience of

the attendant pair but was highly correlated with hatching success, EPOs being twice as common in broods which suffered hatching failures, the main cause of which was infertility. The role of male fertility in determining the success of EPCs and the possible benefits a female may gain from them are discussed.

CHAPTER 5

METRIC VARIATION

This chapter will examine quantitative variation in four morphological characters of the Brackenhurst House Sparrows. Unlike the clearly defined biochemical and DNA sequence polymorphism discussed in previous chapters, analysis of polygenic metric traits is complicated by the interaction of genotype and environment which results in continuously distributed phenotypic variation. Many loci may contribute in varying degrees to the eventual phenotype whilst environmental variation serves to blur the genotypic classes together. Despite the expected difficulties, an attempt to partition genetic and environmental components of phenotypic variation is worthwhile, as several studies have demonstrated the importance of quantitative variation in the evolution, differentiation and social structure of House Sparrow populations.

The classic study of Hermon C. Bumpus (1899) described the effect of a severe winter snowstorm upon the sparrows of the university campus at Providence, Rhode Island. Birds found moribund towards the end of the storm were brought into the laboratory where subsequently half of them died due to its effects. A comparison of the body dimensions of survivors and non-survivors revealed that the more extreme individuals had suffered higher mortality than those close to the population mean, leading Bumpus to propose the concept of stabilizing or centripetal selection. The data have been reanalysed by Johnston *et al.* (1972) and Grant (1972) using more sophisticated statistical techniques. They confirmed that surviving females had lower variances for weight and humeral, sternal and tibiotarsal lengths but found that males had undergone directional selection for increased size. An attempt to repeat the study (by Rising (1970)) revealed an increase in male size between an autumn and spring sample. Since the same birds did not comprise both samples the difference may not be due solely to differential mortality. A larger sample of birds examined by Fleischer and Johnston (1982) revealed a significant increase in male body core with a smaller increase in limb length, whilst females were smaller with dramatically shorter tarsi in

their spring sample. The change in appendicular/core ratio is predictable from Allen's ecogeographic rule.

The ecogeographic rules of Allen and Bergmann correctly predict the differences between North American populations of House Sparrows (Johnston and Selander, 1971 and 1973). Birds in higher latitudes are larger with relatively reduced limb lengths. The differences are highly significant for wing and tarsal lengths and for weight, for which a north-south cline from 20°N to 50°N covers a 6g range in mid-winter bodyweight (Blem, 1973). The introduced Sparrows adapted within a few years of their introduction into North America. Museum specimens from the 1880s are intermediate in body size between British and German House Sparrows which together comprised the founding population, but are significantly smaller than samples from the same localities collected 20 years later (Calhoun, 1947). The morphology of American House Sparrows adapted to the prevailing environmental conditions in less than 30 generations and has subsequently changed little. To achieve this change required heritable genetic variation for body size traits so that selection could increase the frequency of favoured phenotypes.

Mechanisms other than environmental selection may influence the viability and fecundity of Sparrows dependent on particular metric characters. Burke found evidence of significant positive assortative mating for both weight and tail length at the Sutton Bonington population but failed to find any at Brackenhurst, though the sample sizes involved were small. There are few published cases of assortative mating for a metric character in birds. Weak positive assortment has been documented in Darwin's Finches (Boag and Grant, 1978) for a variety of correlated size characters whilst negative assortment has been recorded for beak width of Song Sparrows (*Melospiza melodia*) (Smith and Zach, 1979). Thus the possibility of a genetic basis for this phenomenon is of great interest as, under positive assortment, the additive genetic variance of the metric trait will be enhanced.

Sexual selection for a metric trait has been convincingly demonstrated in the polygynous Long-tailed Widowbird (*Euplectes progne*) (Andersson, 1982) and the monogamous Swallow (*Hirundo rustica*) (Møller, 1988b). In both species tail length manipulations have shown that females prefer to mate with males with long tails. Long tailed Swallows attract mates earlier, increasing their chance of raising further broods, and are less likely to be rejected by a female if they attempt an EPC.

In House Sparrows, bib size, a trait associated with social dominance in several species (Järvi and Bakken, 1984; Rohwer, 1985; Møller, 1987d and e), has been demonstrated to have an important effect on mate choice. D.G. Harper (pers. comm.) found that although it did not predict the outcome of fights it was significantly correlated with nest site quality and the male's ability to attract a second mate polygynously (Møller, 1987d and e, 1988a, 1989a, 1990).

Under sexual selection, a character is favoured by females regardless of their own genotype, whilst assortative mating involves females selecting phenotypes depending on their own. The latter can maintain diversity whereas sexual selection is directional and must be opposed by other forces if the loci involved are to remain polymorphic. Møller has identified some of these in both Swallows and Sparrows. Large bibbed House Sparrows suffer an increased predation rate (Møller, 1989a) while Swallows with artificially elongated tails suffer from impaired foraging efficiency and are less likely to survive through migration to breed in the following year (Møller, 1989b).

A further factor that can affect quantitative genetic variation in metric traits is the pleiotropic effect of enzyme loci or loci in linkage disequilibria with them. One of the earliest papers to report on enzyme polymorphism in the House Sparrow remarkably found that the genotype, at a liver esterase locus, was highly correlated with size as recorded from skeletal measurements (Johnston and Klitz, 1977). The authors

comment that the finding was fortuitous or possibly artefactual. Other studies combining electrophoretic and morphometric data have concentrated on the hypothesis that increased levels of heterozygosity can lead to improved homeostasis during development. The relationship between heterozygosity and homeostasis is discussed at length by Lerner (1954). In short, the variation between allozymes is thought to widen the range of internal physiological conditions over which metabolic processes can continue at high efficiency, resulting in more efficient buffering of developmental processes and decreased environmentally induced variance in metric traits.

Two studies of skeletal variation in the House Sparrow have shown decreased variance in skeletal traits in birds of higher than average heterozygosity (Johnston and Klitz, 1977; Fleischer *et al.*, 1983) although a similar survey of Rufous-collared Sparrows (*Zonotrichia capensis*) found no correlation with genotype (Handford, 1980). Studies of fish and invertebrates have also produced apparently contradictory results (e.g. McAndrews *et al.*, 1982).

The previously cited papers have shown the importance of the environment, genotype and interactions with conspecifics in contributing towards phenotypic variance for quantitative traits in House Sparrow populations. In most cases skeletal characters were used which could not be measured in a long term population study. It was hoped that the analysis of external traits would reveal corresponding phenomena.

Methods

Four measurements were taken of post-fledge birds whenever they were handled. These were weight to the nearest 0.1g, measured with a Pesola spring balance and plastic weighing cone. The maximum chord method (Spencer, 1976) was used for wing length, in which the carpal joint of the wing is pressed firmly against the

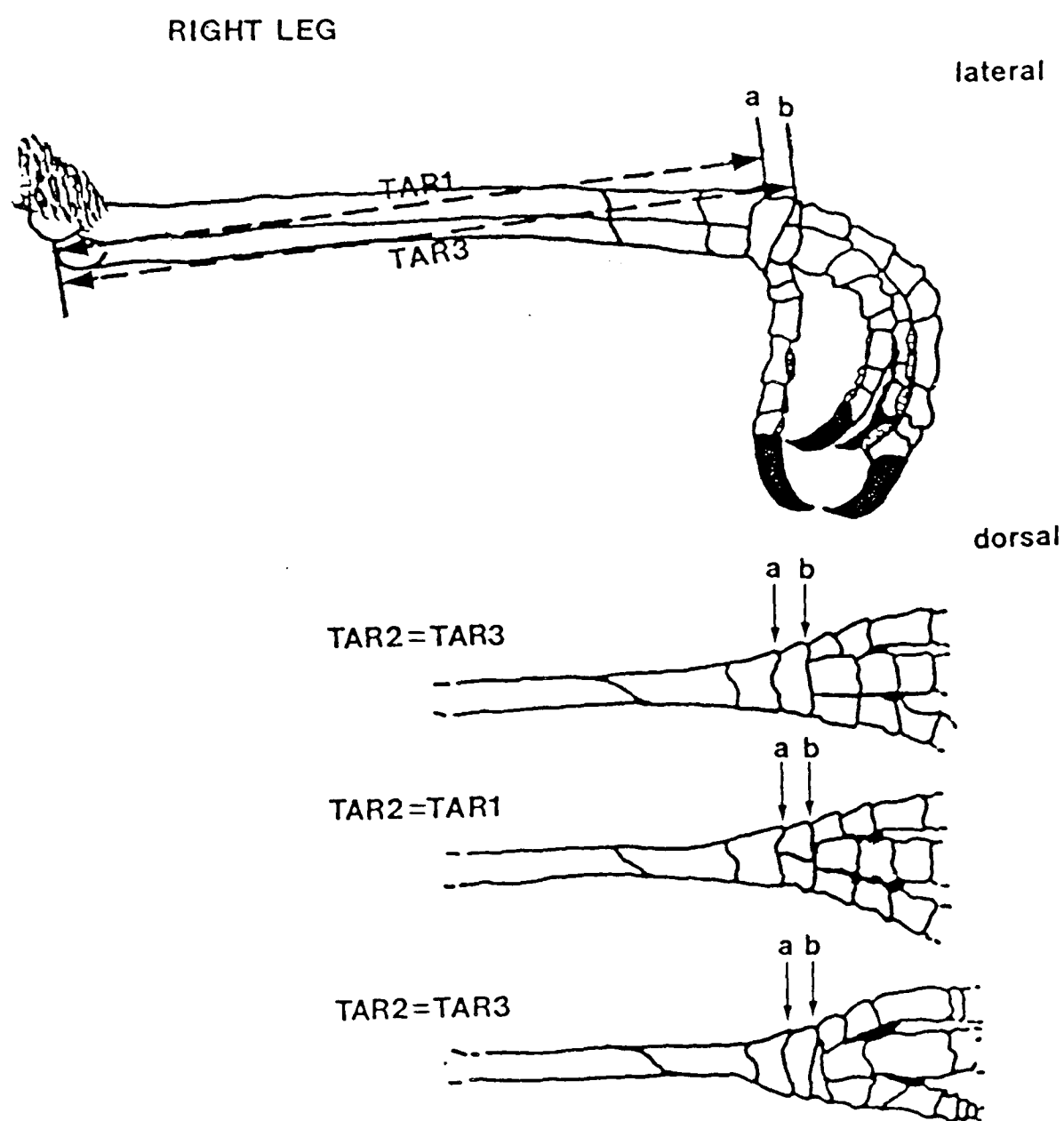
end stop of a ruler whilst the primaries are straightened and flattened by the thumb of the free hand. Tail length was measured using a ruler slid along the underside of the retrices until it came to a stop at the root of the tail. Both plumage measurements were recorded to the nearest millimetre, excluding from the analysis individuals in moult or with damaged feathers. Tarsus measurements (T2) were made with a sliding vernier caliper between the intertarsal notch and the distal edge of the last undivided scute to an accuracy of 0.1mm. In practice it was found that approximately 10% of birds had a distinct split running parallel to the axis of the tarsi across the scale which was usually measured. Measurements were taken to the distal and proximal edges of this scute and the measurement used in subsequent analyses as T2, is indicated in Fig. 5.1 which illustrates the right tarsi of three representative birds.

Individual nestlings within a brood were identified by trimming a specific claw with iris scissors on the first visit after hatching. The nestlings were weighed on this and subsequent visits until ringed at 10 to 15 days of age. At this visit the tarsi were measured using the same technique as described for adults. During subsequent visits to the nestbox, handling of the nestlings was avoided to reduce the risk of premature fledging.

The plumage of juvenile House Sparrows is very similar to that of adult females. However, they can be distinguished by their smoother plumper tarsi, remnants of the yellow fleshy gape flanges and unworn feathers. The latter are completely replaced by the yearling plumage during the post-juvenile moult which commences four to six weeks after fledging and continues for two to three months. The first signs of sexual dimorphism appear one month after the onset of moult when males acquire chestnut scapulars and the distinctive head pattern. Prior to this, some males have a greyer throat and whitish post-ocular spot even as nestlings. Juvenile females are more problematic as they cannot be sexed with confidence until October when the youngest males have started to acquire their adult plumage.

Figure 5.1

Representative right tarsi from three House Sparrows indicating the measurement used as T2 in the analyses



For the purpose of subsequent analyses and discussions, juveniles become yearlings in October after the post-juvenile moult and then full adults the following year. Likewise, years run from the 1st of October in the previous calendar year and represent the period during which a bird possesses the plumage it will carry during that breeding season.

The presence of both freshly moulted juveniles and "adults" from August onwards necessitates the grouping of an increasing number of individuals in a heterogeneous age class. The majority of these will in fact be young birds as ringing studies have shown that their numbers peak at this time of year before declining rapidly due to their higher mortality rates until approximately equal proportions of yearlings and adults are attained by the end of the winter (Summers-Smith, 1988).

By August, the majority of survivors from the previous year's cohort have been ringed. Hence it is much more likely that an unmarked bird in adult plumage after September is a yearling than a previously uncaptured adult. In total, only 16% of the adults used in the metric analyses were originally captured as juveniles. Hence the age of most birds was estimated on this premise. Individuals of known and uncertain age were initially analysed separately.

The possible sources of metric variation were examined using all measurements of birds collected between 1st October 1985 and 1st March 1989. Intensive netting was resumed in May 1985 having lapsed since 1982. Initially birds were measured either by D. Walters or J.H. Wetton. However, due to potentially differing techniques, only data collected after September 1985 when J.H. Wetton began to measure all birds with a standard technique were used in subsequent analyses. In addition, birds ringed prior to this date included the majority of adults within the Brackenhurst site and this aided the estimation of age for birds caught at later dates.

RESULTS

A multifactorial ANOVA was used to partition variance by age, year and month. The sexes were analysed separately because of sexual dimorphism for all four metric traits. Age was subdivided as follows:-

AGE	CATEGORY
Known juveniles and pulli	0.0
Known yearlings	1.0
Assumed yearlings	1.5
Known 2nd year adults	2.0
Assumed 2nd year adults	2.5
	Etc.

The preliminary breakdown of important factors is shown in Table (5.1) which includes all measurements of birds in adult plumage. Tarsus length shows no temporal variation but significant differences between some age classes. Wing and tail length are much more highly correlated with age, most of the assigned variance being accounted for by an increase in plumage size between yearlings (1.0 and 1.5) and older birds (≥ 2.0). Plumage varied to a lesser degree between months and years. A trend towards increased mean plumage length between years was noted (see later). This contributed to some of the variation between months as January and February samples were only obtained in 1989 when birds were, on average, larger. A small underlying decrease in feather length was also apparent through the spring and summer which was presumably due to abrasion and possibly in the late summer, through accidental inclusion of individuals which had entered the moult. The remaining temporal variation showed no clear pattern.

Changes in weight were independent of age, but demonstrated large fluctuations between months. Both sexes held a relatively stable over-winter weight between October and May followed by a decline during the breeding season and a rapid rise corresponding with the fledging of the last broods and the movement of adults into the

Table 5.1

The initial multi-variate ANOVA of all metric data collected at Brackenhurst between October 1985 and February 1989 of "adult" birds (i.e. yearlings and older)

Sex	Metric	F-Ratio and significance level			
		Age df = 1	Year df = 3	Month df = 10	Residual d.f.
Male	T2	9.55 ***	1.45	0.97	508
	Wing	49.48 *****	1.81	2.16 *	501
	Tail	84.87 *****	4.28 ***	1.45	495
	Weight	0.63	6.44 *****	4.44 *****	504
Female	T2	5.14 *	0.54	1.21	315
	Wing	7.03 **	2.42	1.63	314
	Tail	25.10 *****	4.89 *****	1.92 *	307
	Weight	2.90	4.30 ***	2.32 ***	316

* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.005$, **** = $P < 0.001$, ***** = $P < 0.0001$

ripening grain fields. After the harvest the adults return to the farm to reoccupy nest sites and decline slightly in weight to the over-winter level. The simple measure of excluding weight data collected between June and September removed most of the temporal variation from the analysis.

The data were re-examined after averaging the metric values for each individual within years (excluding the summer months for weight) and dividing birds by age into yearlings (1.0 and 1.5) and older birds (≥ 2.0). Post-fledge juveniles (0.0) were also examined (Tables 5.2a, 5.2b and 5.3). No difference in tarsus length was found between yearlings and adults, other than males in 1987, but both age categories were significantly larger than the juveniles. Mean juvenile tarsus length increased between 1986 and 1988 ($F_{2,130} = 3.32$, $P < 0.05$) and this was mirrored by an increase in yearling tarsus length between 1987 and 1989 (Table 5.3). Yearlings in 1987 were the smallest of all four years (mean of males = 18.375 ± 0.957 mm (Table 5.2a) and females 18.335 ± 0.770 mm (Table 5.2b)) presumably because the juvenile cohort from which they were recruited was unusually small. No heterogeneity was observed in the full adult classes. The presence of many birds in consecutive adult samples buffers the mean value against changes in recruit size.

No significant differences were found between years for the two plumage variables in any age or sex class other than full adult tail length in males which increased steadily over the four years. However, dramatic differences were observed between yearlings and full adults in each year for tail lengths (all $P < 0.001$ for males and $P < 0.005$ for females) and in 1988 for wing length in both sexes and for males only in 1989.

The two "adult" age classes had similar mean weights in each year. However, yearlings were lighter than full adults in 1986 and significantly so for females ($F = 12.58$, $P < 0.001$). Full adult weight did not vary between years but some

Table 5.2a

Comparison of mean yearling and full adult metrics by year for females

		Yearling		2nd Year +		F	Sig
Metric	Year		N		N		
T2	1986	18.477 ± 0.755	31	18.751 ± 0.649	38	3.24	N.S.
	1987	18.335 ± 0.770	37	18.625 ± 0.871	33	2.20	N.S.
	1988	18.490 ± 0.688	35	18.531 ± 0.980	27	0.04	N.S.
	1989	18.780 ± 0.839	15	18.372 ± 0.816	12	1.61	N.S.
Heterogeneity		F _{3,134} = 1.26 N.S.		F _{3,106} = 0.79 N.S.			
Wing	1986	74.631 ± 1.840	51	74.919 ± 1.588	37	0.59	N.S.
	1987	75.014 ± 1.618	37	75.705 ± 1.571	35	3.37	N.S.
	1988	74.623 ± 1.615	36	75.542 ± 1.674	24	4.53	*
	1989	75.200 ± 1.740	15	75.875 ± 2.268	12	0.77	N.S.
Heterogeneity		F _{3,135} = 0.76 N.S.		F _{3,104} = 1.75 N.S.			
Tail	1986	55.260 ± 1.624	50	56.289 ± 1.642	38	8.59	***
	1987	55.302 ± 1.663	37	56.343 ± 1.365	35	8.37	***
	1988	55.838 ± 1.407	36	56.993 ± 1.477	24	9.33	***
	1989	55.633 ± 1.043	15	57.417 ± 1.893	12	9.70	***
Heterogeneity		F _{3,134} = 1.22 N.S.		F _{3,105} = 2.45 N.S.			
Weight	1986	27.471 ± 1.864	51	28.855 ± 1.723	37	12.58	****
	1987	27.973 ± 1.448	37	27.904 ± 2.026	35	0.03	N.S.
	1988	28.332 ± 1.973	36	28.619 ± 1.686	27	0.37	N.S.
	1989	28.753 ± 1.669	15	29.114 ± 1.099	12	0.41	N.S.
Heterogeneity		F _{3,135} = 2.82 *		F _{3,107} = 2.34 N.S.			

* = P < 0.05, ** = P < 0.01, *** = P < 0.005, **** = P < 0.001

Table 5.2a

Comparison of mean yearling and full adult metrics by year for females

		Yearling		2nd Year +		F	Sig
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	1989	18.780 ± 0.839	15	18.372 ± 0.816	12	1.61	N.S.
Heterogeneity		F _{3,134} = 1.26 N.S.		F _{3,106} = 0.79 N.S.			
Wing	1986	74.631 ± 1.840	51	74.919 ± 1.588	37	0.59	N.S.
	1987	75.014 ± 1.618	37	75.705 ± 1.571	35	3.37	N.S.
	1988	74.623 ± 1.615	36	75.542 ± 1.674	24	4.53	*
	1989	75.200 ± 1.740	15	75.875 ± 2.268	12	0.77	N.S.
Heterogeneity		F _{3,135} = 0.76 N.S.		F _{3,104} = 1.75 N.S.			
Tail	1986	55.260 ± 1.624	50	56.289 ± 1.642	38	8.59	***
	1987	55.302 ± 1.663	37	56.343 ± 1.365	35	8.37	***
	1988	55.838 ± 1.407	36	56.993 ± 1.477	24	9.33	***
	1989	55.633 ± 1.043	15	57.417 ± 1.893	12	9.70	***
Heterogeneity		F _{3,134} = 1.22 N.S.		F _{3,105} = 2.45 N.S.			
Weight	1986	27.471 ± 1.864	51	28.855 ± 1.723	37	12.58	****
	1987	27.973 ± 1.448	37	27.904 ± 2.026	35	0.03	N.S.
	1988	28.332 ± 1.973	36	28.619 ± 1.686	27	0.37	N.S.
	1989	28.753 ± 1.669	15	29.114 ± 1.099	12	0.41	N.S.
Heterogeneity		F _{3,135} = 2.82 *		F _{3,107} = 2.34 N.S.			

* = P < 0.05, ** = P < 0.01, *** = P < 0.005, **** = P < 0.001

Table 5.2b

Comparison of mean yearling and full adult metrics by year for males

		Yearling		2nd Year +		F	Sig
Metric	Year		N		N		
T2	1986	18.788 ± 0.794	74	18.857 ± 0.690	67	0.30	N.S.
	1987	18.375 ± 0.957	33	18.828 ± 0.930	52	4.67	*
	1988	18.826 ± 0.688	70	18.860 ± 0.942	36	0.04	N.S.
	1989	18.743 ± 0.988	19	18.902 ± 0.701	27	0.41	N.S.
Heterogeneity		F _{3,190} = 2.57 *		F _{3,178} = 0.05 N.S.			
Wing	1986	77.839 ± 1.874	73	78.082 ± 1.814	67	0.60	N.S.
	1987	77.490 ± 1.897	33	78.141 ± 1.692	51	2.69	N.S.
	1988	77.439 ± 1.583	69	78.561 ± 1.600	35	11.59	****
	1989	77.711 ± 1.610	19	78.827 ± 1.329	27	6.61	*
Heterogeneity		F _{3,190} = 0.70 N.S.		F _{3,176} = 1.71 N.S.			
Tail	1986	57.034 ± 1.865	72	58.490 ± 1.703	67	23.00	****
	1987	57.333 ± 1.717	33	58.596 ± 1.498	52	12.79	****
	1988	57.626 ± 1.832	70	58.915 ± 1.286	35	13.87	****
	1989	57.667 ± 1.404	18	59.423 ± 1.188	27	20.39	****
Heterogeneity		F _{3,189} = 1.49 N.S.		F _{3,177} = 2.8 *			
Weight	1986	28.171 ± 1.979	73	28.632 ± 1.832	68	2.10	N.S.
	1987	28.746 ± 1.933	33	28.454 ± 1.564	52	0.59	N.S.
	1988	29.174 ± 1.712	70	29.440 ± 1.810	36	0.55	N.S.
	1989	28.819 ± 1.663	19	28.780 ± 1.833	26	0.01	N.S.
Heterogeneity		F _{3,191} = 3.56 *		F _{3,178} = 2.45 N.S.			

Table 5.3

Change in metrics with increasing age of three Brackenhist cohorts

Metric	Year	Nestlings	N	Juveniles	N	Yearlings	N	N → J	J → Y
T2	1986	18.167 ± 0.065	193	18.269 ± 0.130	52	18.408 ± 0.091	98	0.52 N.S	0.78 N.S
	1987	18.305 ± 0.063	165	18.606 ± 0.161	34	18.717 ± 0.066	154	3.69 N.S	1.00 N.S
	1988	18.326 ± 0.107	58	18.771 ± 0.156	47	18.759 ± 0.156	34	5.89 *	0.03 N.S
Heterogeneity		F _{2,413} = 1.48 N.S.		F _{2,130} = 3.32 *		F _{2,283} = 4.43 *		F Sig	F Sig
Weight	1986	22.892 ± 0.198	199	26.405 ± 0.345	51	28.271 ± 0.176	101		
	1987	23.675 ± 0.205	175	28.096 ± 0.287	37	28.657 ± 0.166	153		
	1988	24.438 ± 0.382	64	27.989 ± 0.337	46	28.790 ± 0.281	34		
Heterogeneity		F _{2,435} = 8.47 ****		F _{2,131} = 8.53 ****		F _{2,285} = 1.57 N.S.			

* = P<0.05, ** = P<0.01, *** = P<0.005, **** = P<0.001

Nestling measurements were made on the day of ringing at 11-15 days of age. Juveniles were measured after fledging but before the completion of the post-juvenile moult. The yearling mean was derived from all measurements made after completion of the post-juvenile moult but before the following October. The data was tested for heterogeneity within and between age classes.

heterogeneity was apparent in yearlings (males $F = 3.56$ $P < 0.05$ and females $F = 2.82$ $P < 0.05$) being lowest in 1986.

The sources of metric variation

Many factors may produce differences in size between individuals within a population. The importance of genotype in determining size is dependent on the heritability of the trait. However, before heritabilities can be determined the effect of temporal and environmental variation must be examined. Two approaches may be used. Either many individuals are sampled simultaneously to analyse differences due to age by sampling a variety of cohorts, or repeat measurements of the same individuals in different months or years can be taken.

The former approach, although it may reveal dramatic differences between cohorts, can provide only limited information as to how they initially arose. Longitudinal samples are less common as they require observation of a study population over several years with repeated non-destructive sampling. Workers who have carried out such studies have often sacrificed birds surviving the period of interest to obtain skeletal measurements. Clearly such steps could not be taken in a population where differences in reproductive success were of primary interest.

Measurement error will contribute towards the variance for all four traits and will be the most important environmental factor for tarsus length once growth is complete. Age related changes in plumage length may occur at the yearly moult and abrasion of feathers is known to be a likely confounding variable. Weight is expected to show the greatest temporal variation as it is known to fluctuate between and even within days (O'Connor, 1972).

To investigate the degree of temporal variation and amount of measurement error associated with the metric characters, both inter- and intra-year repeatabilities were calculated. These relate the magnitude of change between repeat measurements of an individual to the range of sizes found within the population by dividing the between-individual variance component by the sum of the within- and between-component estimates (Lessels and Boag, 1987). The sexes are analysed separately because dimorphism will artificially inflate the between-individual component, thus raising the repeatability. Table 5.4 summarises the differences in size between males and females. Oddly despite the lack of age related changes in weight, yearlings are sexually dimorphic for weight whilst full adults are not.

The repeatabilities are given in Tables 5.5 and 5.6. Repeatability of tail length was higher for intra-year comparisons of individual measurements than for comparisons of annual means between years, whilst wing length had a higher inter-year repeatability. Values for weight and tarsus were similar both within and between years, and are comparable with published repeatabilities of these measurements in other species (references in Boag and van Noordwijk, 1987).

The difference between repeat measurements (Tables 5.5 and 5.6) can provide an indication of whether measurement error or a systematic change is involved in reducing repeatabilities from unity. Tarsus length which has the highest repeatability has a mean change between measurements which is not significantly different from zero either within or between years. This is not surprising since adult size is attained towards the end of the nestling period. In the absence of further growth, within individual variation will be due solely to measurement error.

Likewise weight is a highly repeatable measurement in both males and females within and between years. In both tables measurements collected between May and

Table 5.4

Sexual Dimorphism

Age	Metric	Male	N	Female	N	F	Sig
Yearling	T2	18.728 ± 0.058	196	18.473 ± 0.064	138	8.36	***
	Wing	77.625 ± 0.126	194	74.797 ± 0.145	139	215.38	*****
	Tail	57.359 ± 0.129	193	55.461 ± 0.131	138	101.10	*****
	Weight	28.854 ± 0.138	149	28.223 ± 0.173	102	8.23	***
Full Adult	T2	18.879 ± 0.067	132	18.600 ± 0.084	87	6.81	**
	Wing	78.276 ± 0.149	129	75.424 ± 0.183	87	147.10	*****
	Tail	58.742 ± 0.129	130	56.582 ± 0.162	87	106.79	*****
	Weight	28.725 ± 0.146	130	28.544 ± 0.185	85	0.44	N.S.

* = P <0.05, ** = P <0.01, *** = P <0.005, **** = P <0.001, ***** = P <0.0001

Table 5.5**Inter-Year Repeatabilities**

Metric	Sex	F-Ratio	DF	Repeat-ability	Average Increase between repeat measurements	Deviation from no change
Tail	Male	4.41	37,51	0.592	$0.517 \pm 0.167\text{mm}$	$P < 0.01$
	Female	3.48	18,22	0.332	$0.460 \pm 0.325\text{mm}$	$P > 0.1$
Wing	Male	4.72	37,51	0.613	$0.353 \pm 0.167\text{mm}$	$P \sim 0.05$
	Female	6.09	17,21	0.701	$0.653 \pm 0.230\text{mm}$	$P < 0.05$
Weight	Male	9.15	59,83	0.773	$0.28 \pm 1.18\text{g}$	$P > 0.5$
	Female	4.13	36,40	0.600	$2.18 \pm 2.37\text{g}$	$P > 0.1$
T2	Male	9.20	68,91	0.788	$0.001 \pm 0.050\text{mm}$	$P > 0.5$
	Female	11.39	45,43	0.927	$0.008 \pm 0.066\text{mm}$	$P > 0.5$

Inter-year repeatabilities for plumage traits were determined from multiple measurements of full adults (i.e. 2 year old +), all adults including yearlings were included in the analysis of weight and T2. The significance of the change between repeat measurements was calculated using Wilcoxons single sample test.

Table 5.6**Intra-Year Repeatabilities**

Metric	Sex	F-Ratio	DF	Repeat-ability	Average Increase between repeat measurements	Deviation from no change
Tail	Male	4.89	80,94	0.652	-0.151 ± 0.131mm	P>0.1
	Female	2.47	40,46	0.414	-1.000 ± 0.240mm	P<0.001
Wing	Male	4.17	82,97	0.601	-0.198 ± 0.140mm	P>0.1
	Female	2.00	44,52	0.317	-0.695 ± 0.248mm	P<0.005
Weight	Male	8.08	61,68	0.774	-0.173 ± 0.129g	P>0.1
	Female	6.62	22,25	0.734	-0.467 ± 0.285g	P>0.1
T2	Male	6.45	85,99	0.717	0.091 ± 0.047mm	P>0.05
	Female	8.66	45,52	0.786	-0.098 ± 0.077mm	P>0.1

Intra-year repeatabilities for metric traits were determined from multiple measurements of all adults (i.e. yearlings and older birds).

September were excluded from the calculations because of the dramatic weight fluctuations which occur during the breeding season.

Intra-year repeatabilities were calculated using all “adult” birds (age class ≥ 1.0). Male plumage length did not change within years ($P > 0.1$ for both wing and tail), whereas females showed a significant mean decrease between measurements with time for tail ($-1.000 \pm 0.240\text{mm}$, $N = 57$, $P < 0.001$) and wing length ($-0.695 \pm 0.248\text{mm}$, $N = 59$, $P < 0.005$). The difference between the sexes might arise because females are more likely to abrade their plumage whilst making frequent visits to, and spending more time in, the nest. The inter-year correlations for plumage traits were calculated using only full adults (≥ 2.0) and show significant increases for male tail length ($0.517 \pm 0.167\text{mm}$, $N = 67$, $P < 0.01$), and male and female wing length ($0.353 \pm 0.167\text{mm}$, $N = 67$, $P < 0.05$ and $0.653 \pm 0.230\text{mm}$, $N = 24$, $P < 0.05$ respectively). This strongly indicates that growth between moults is responsible for the differences between age classes.

The hypothesis that growth rather than selection or some other process was responsible for the difference between yearlings and second year adults was examined using the mean change between the age classes (Table 5.7). The known age group revealed highly significant increases in tail length of between 1 and 1.5mm ($P < 0.001$), wing length increased but the change was not significant. However, the combined data set (1.0 and 1.5) gave a significant increase in wing length (0.358 ± 0.169 , $N = 81$ males, $P < 0.05$, and $0.614 \pm 0.219\text{mm}$, $N = 41$ females, $P < 0.05$). Clearly growth of longer feathers at successive moults could account for much of the variance between age classes, but other factors could be involved.

Dispersal occurs mainly with the break up of the grain field flocks in September but a few juveniles change area in the early spring if they have been unable to find a suitable nest site. The second wave of dispersal occurred after most of the yearling

Table 5.7

Mean increase from yearling size of adults recaptured in their first post-yearling plumage (i.e. as 2 year olds)

a)

Sex	Metric	Mean increase \pm SE	Range	Deviation from zero
Male N = 81	T2	$-0.004 \pm 0.065\text{mm}$	-1.4, +2.7	N.S.
	Wing	$0.358 \pm 0.169\text{mm}$	-5, +4	*
	Tail	$0.637 \pm 0.145\text{mm}$	-2.5, +4	****
	Weight	$-0.001 \pm 0.151\text{g}$	-4, +3.8	N.S.
Female N = 41	T2	$-0.005 \pm 0.080\text{mm}$	-1.15, +2.25	N.S.
	Wing	$0.614 \pm 0.219\text{mm}$	-2, +6	*
	Tail	$1.073 \pm 0.226\text{mm}$	-2, +5	****
	Weight	$0.550 \pm 0.216\text{g}$	-2.05, +4.05	*

b)

Male N = 34	T2	$0.069 \pm 0.121\text{mm}$	-1.4, +2.7	N.S.
	Wing	$0.397 \pm 0.297\text{mm}$	-5, +3	N.S.
	Tail	$1.115 \pm 0.232\text{mm}$	-2, +4	****
	Weight	$0.031 \pm 0.208\text{g}$	-2.5, +2.9	N.S.
Female N = 25	T2	$-0.066 \pm 0.112\text{mm}$	-0.9, +2.25	N.S.
	Wing	$0.620 \pm 0.323\text{mm}$	-2, +6	N.S.
	Tail	$1.440 \pm 0.309\text{mm}$	-2, +5	****
	Weight	$0.811 \pm 0.281\text{g}$	-2.05, +4.05	*

* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.005$, **** = $P < 0.001$

Data set (a) includes birds assigned as probable yearlings (e.g. age class 1.5). These are omitted from subset (b) which consists of birds rung as nestlings or juveniles which were then captured in the next two years.

measurements had been collected. If smaller birds dispersed then the birds which remained to breed would be longer than average. A similar effect would be seen if selection favoured large individuals. However, if growth was the most important variable then the mean size of yearlings which were not recaptured or resighted in later years would be similar to those birds which disappeared from the population, either through dispersal or death.

All yearlings (1.0 and 1.5) measured in 1986 and 1987 were compared with those known to have survived until after the next moult (Table 5.8). Yearlings from 1988 and 1989 were not used to avoid excluding individuals which were present in the population but avoided capture in the next year. None of the four traits showed a significantly higher mean for those birds destined to remain in the population, but larger birds may have been favoured as only female weight gave a lower value. If we compare the increases known to occur with growth with the difference between all sampled yearlings and second year adults, we note a very close correspondence (Table 5.9) which provides a much better fit than the differential survival hypothesis.

Differences between the plumage of yearling and older House Sparrows have been recorded from large scale morphometric samples collected by culling late autumn flocks (references in Summers-Smith, 1988). Birds were aged by the extent of skull ossification which is not complete until the bird is approximately seven months old (Nero, 1951). However, since only a single sample was collected, it was not possible to determine the basis of the difference.

By resampling the Brackenhurst population it has been possible to show that plumage length increases between moults and that differential mortality or dispersal are probably not involved. There remains the possibility of a methodological bias, the most probable cause of which would be the inclusion of late moulting juveniles in the yearling sample. This is easily refuted by examination of the mean yearling and adult

Table 5.8

Sex	Metric	All Yearlings	N	Survivors Only	N	F	Sig	Increase
Male	T2	18.661 ± 0.835	107	18.735 ± 1.720	33	0.18	N.S.	0.074mm
	Wing	77.730 ± 0.183	106	78.217 ± 0.306	33	1.74	N.S.	0.487mm
	Tail	57.128 ± 0.177	105	57.287 ± 0.231	32	0.21	N.S.	0.159mm
	Weight	28.350 ± 1.917	106	28.701 ± 2.669	33	0.88	N.S.	0.351g
Female	T2	18.417 ± 0.810	88	18.446 ± 1.606	24	0.03	N.S.	0.029mm
	Wing	74.792 ± 0.187	88	75.292 ± 0.288	24	1.66	N.S.	0.500mm
	Tail	55.278 ± 0.175	87	55.521 ± 0.347	24	0.41	N.S.	0.243mm
	Weight	27.682 ± 1.824	88	27.442 ± 2.935	24	0.39	N.S.	-0.240g

Comparison between yearlings known to have survived until their second adult year and all yearlings measured in 1986 and 1987.

Table 5.9

Sex	Metric	Observed change due to growth (see Table 5.7)	Difference between yearling and full adult age class (see Table 5.2a & 2b)	Difference between “survivors” and “non-survivors” (see Table 5.8)
Male	Wing	0.397 mm	0.651 mm	0.487 mm
	Tail	1.115 mm	1.349 mm	0.159 mm
Female	Wing	0.620 mm	0.632 mm	0.500 mm
	Tail	1.440 mm	1.119 mm	0.243 mm

The relative increase in plumage length between yearling and full adult age classes associated with growth and mortality

plumage length within each month. In many instances the October sample was slightly smaller than that from November but the difference clearly could not have contributed significantly to the difference between age classes.

Throughout this section the known and assumed yearling classes have frequently been pooled to increase the sample sizes. This seemed to be justified both on demographic grounds and on the morphological similarity of the two classes for the age related traits of wing and tail length. Analysis of variance was used to compare the three age classes 1.0, 1.5 and 2.0. If the 1.5 age class is comprised largely of yearlings then the morphological similarity with true yearlings will be greater than with known second year birds. This appears to be true for tail length (Fig. 5.2) and is confirmed by the significant proportion of the variance accounted for by category between the 1.5 and 2.0 groups compared with the 1.0 and 1.5 age classes (Table 5.10), thus pooling seems to be justified.

Possible ultimate causal factors in age related variation

We have seen that much of the variance between the yearling and full adult cohorts in plumage length is accounted for by growth at the second annual moult and that similar increases occur at subsequent moults. What is the reason for the increase?

House Sparrows replace all their primaries and retrices at each annual moult (Summers-Smith, 1963). However, some passerines have an arrested moult if they enter a period of protein stress during which insufficient protein is obtained from the diet to allow new feather growth (Gosler, in press). A close correlation between low protein reserves indicated by poor pectoral muscle condition and number of unmoulted greater coverts has been found in Great Tits (*Parus major*). In a Belgian Great Tit population a further correlation was found with fledging date in females (Dhondt, 1973). This was examined in yearlings which were ringed as nestlings. Birds which

Figure 5.2

The tail length distributions of known yearlings and second year adults compared with those assumed to be yearlings. The mean of each age class is indicated

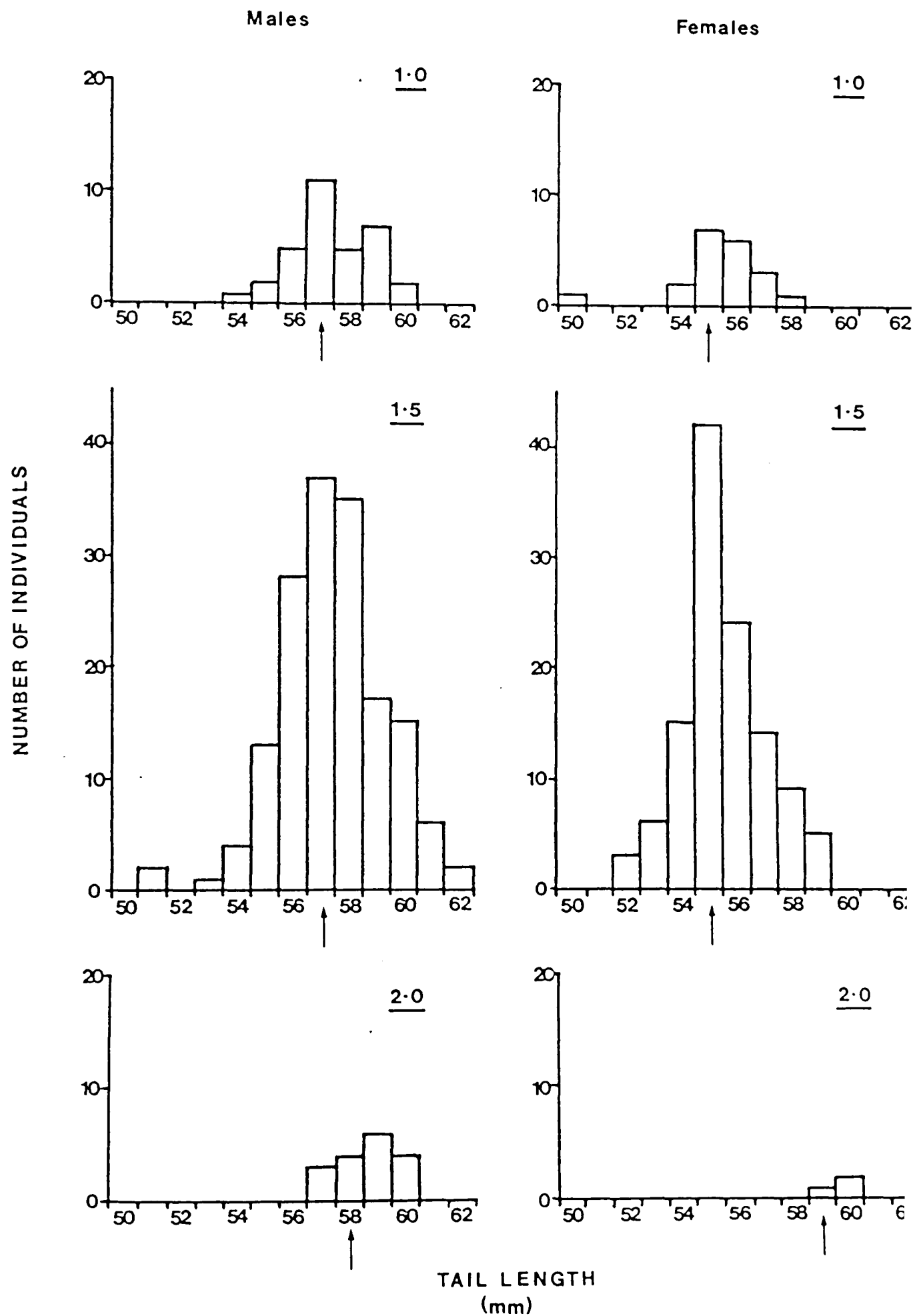


Table 5.10

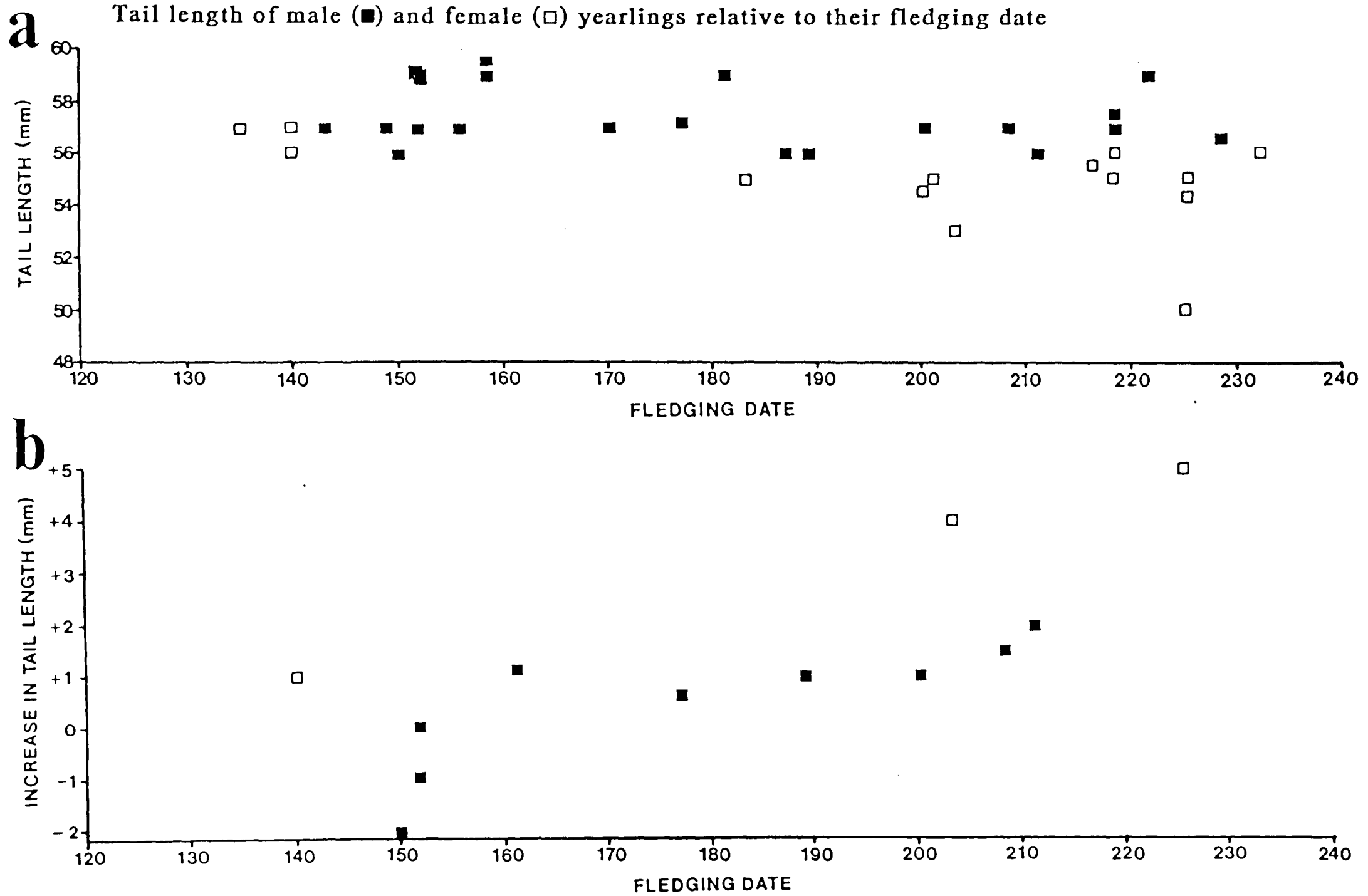
Justification of pooling birds of uncertain age (1.5) with the yearling age class by analysis of variance

Sex	Age Class	Mean tail length	SE	N	
Female	1.0	55.325	± 0.361	20	$F_{1,136} = 0.18$ N.S. $F_{1,119} = 22.33$ P <0.001
	1.5	55.484	± 0.140	118	
	2.0	59.667	± 0.333	3	
Male	1.0	57.333	± 0.245	33	$F_{1,189} = 0.07$ N.S. $F_{1,173} = 6.64$ P <0.05
	1.5	57.419	± 0.142	158	
	2.0	58.559	± 0.246	17	

fledged later in the year had significantly shorter tails ($t = 2.67$, $P < 0.05$, $N = 24$) when length was regressed on fledging date having removed sexual dimorphism as a factor (Fig. 5.3a). The effect was not due to the inclusion of moulting individuals from late broods as omission of measurements before January gave a similar picture. It is likely that fledglings from later broods might have acquired less substantial protein reserves prior to commencement of the post-juvenile moult since they had only just gained independence from their parents.

It is interesting to note that there is no correlation between weight ($t = 1.31$, N.S.) or tarsus length ($t = 0.78$, N.S.) and fledging date although there are significant fluctuations in both with date within year (see later), possibly due to unpredictable variation in the food supply. The nestling data implies that birds fledging late in the year are no smaller in skeletal proportions but may lack resources during the post-juvenile moult. If this is the case these birds should be capable of growing a plumage more in keeping with their true body size at later moults. This was examined by comparing the increase in length between yearling and first full adult plumages for birds of known fledging date. A significantly greater increase was found for those birds which had fledged late and acquired a short yearling plumage ($t = 3.34$, $P < 0.01$) (Fig. 5.3b). It is not known for certain that some Brackenhurst juveniles do not retain old abraded feathers, thereby appearing to grow a short plumage. If arrested moulting was the cause of the dramatic difference between the age classes it would surely have been noted by many authors. However, Summers-Smith's (1988) review lists only two isolated reports from Senegal and Texas. Thus, it appears that the moult proceeds as expected but the growth of all feathers is slightly retarded. Continued growth at future moults may be partly accounted for by greater growth of birds which had unusually short yearling plumages due to late fledging. Improved protein reserves in the more experienced birds allows attainment of the normal length plumage.

Figure 5.3



Status signalling is an alternative explanation for increased plumage length with successive moults. Selander and Johnston (1967) examined large samples of yearling and full adult male Sparrows and found that some yearling males possessed a facial pattern intermediate between that of juveniles and adult males. It was shown that the control of facial pattern is not influenced by hormone titres but is closely related to age at the commencement of the post-juvenile moult. Male fledglings from early broods are relatively older when they enter the moult and develop the normal adult markings. However, birds which enter the moult shortly after fledging develop a more juvenile-like mask with less black feathering. If these feathers are plucked early in December they regenerate as normal black adult feathers, the feather papillae having matured in the intervening period. A correlation was noted between blackness of the lores (a possible indication of fledging date) and wing and tail length. This corresponds with the findings from the Brackenhurst population where the youngest yearlings develop the shortest plumages.

Selander and Johnston (1967) hypothesized that the retention of a less conspicuous juvenile-like plumage was related to the difficulties experienced by late fledging yearlings in acquiring nest-sites and mates. There is some evidence that late fledging males have less chance of finding nest sites in competition with older birds. Following the break up of the grain field flocks, adults return to the nests which they occupied previously and the slightly later returning juveniles compete for the remaining sites. Summers-Smith (1963) suggests that the yearling birds who acquire nest sites or replace lost mates in October originate from the earlier broods and the pairs formed just before breeding commences are from later broods. Many of these fail to breed at all. Selander and Johnston (1967) suggest that the physiological mechanism controlling mask plumage colour has been adjusted by selection so that it is directly related to the probability of successful reproduction in the first year. Those birds with the least chance of reproducing in their first year acquire a duller plumage which reduces the frequency of agonistic behavioural interactions and increases the chances of survival by

rendering the birds less conspicuous to predators. Some supporting evidence for this hypothesis is given by studies of badge size in male sparrows.

The darkness of the mask is correlated with the length of the pale grey edging to the feathers which conceal the black bib until they abrade away (Selander and Johnston, 1967). Thus birds with pale masks conceal their bibs more completely. Bib size is positively correlated with social dominance (Møller, 1987d), the number of agonistic encounters (Møller, 1987e) and the ability to acquire a nest site and mate (Møller, 1989a, 1990). Also males with large conspicuous bibs suffer heavier predation by Sparrowhawks (*Accipiter nisus*) (Møller, 1989a).

There is some evidence that status signalling may be involved in the colouration of yearling plumage and that it is related to tail length which is known to be affected by age at the post juvenile moult. Therefore the possibility remains that plumage length itself is related to status signalling, particularly as the wings and tail are held in such a way that their size is obvious to other birds during threat and solicitation displays (Summers-Smith, 1963). However, there is no evidence that birds fledging from earlier broods or yearlings with long tails live longer (both $P > 0.5$), and so those of subordinate status do not suffer in terms of survival but may have reduced reproductive success.

One metric trait does appear to be correlated with survival. Tarsus length attains its adult size by the time of ringing at 10-14 days of age. This is shown by the high repeatabilities of nestlings retrapped as adults of 0.834 (0.720 for $N = 19$ males and 0.986 for $N = 7$ females) which compares favourably with the repeatability of adult measurements. The mean change between nestling and adult length is $-0.0338 \pm 0.0933\text{mm}$ which does not vary significantly from zero ($P > 0.5$, Wilcoxon single sample test). However, the nestling age class in each year is significantly smaller than the juveniles implying that some of the smallest nestlings are not recruited into the

juvenile population (Table 5.3). Either selective mortality removes these birds or they are more likely to disperse rapidly from the population.

The latter hypothesis is most unlikely as dispersers tend to be the largest birds for most skeletal traits (Fleischer *et al.*, 1984) and no difference was noted between yearlings ringed on site as nestlings and those caught as juveniles in the grain field flocks which presumably include birds dispersing from other colonies. Higher mortality of small recently fledged juveniles has been recorded in the Great Tit (Garnett, 1981) where survival is closely linked with tarsus length. In this species the higher dominance rank of larger birds is responsible for the differential mortality. In House Sparrows the target of selection is unknown.

There is also variation between years in mean juvenile tarsus length which is tracked one year later by a similar shift in yearling size as recruitment occurs. These do not reflect changes in the size of the breeding adults as this value barely changes. Hence it must reflect some form of environmental variation. Controlled laboratory experiments which involved manipulating the diet of nestling Zebra Finches (Boag, 1987) produced permanent differences in tarsus length between individuals fed on high and low protein diets. Similar shifts have been observed in wild populations of the Finch *Geospiza fortis* (Boag, 1983). Therefore differences in tarsus length between years probably reflect the food supply available during the nestling period.

As well as the rapid increase in mean tarsus length between nestling and juvenile age classes, a significant regression of increasing tarsus length on age was noted for adult males ($t = 3.00$, $P = <0.005$, $N = 283$) and yet there is no difference between the two largest age categories, i.e. yearlings and full adults. Much of the slope is accounted for by the high weighting given to the few males which lived for more than four years. These males may, however, be representatives of unusually long limbed cohorts produced in years when food supplies were abundant before tarsus

measurements were collected systematically. It may be significant that the smallest nestlings (the 1986 cohort) were produced at the highest population density at Brackenhurst (see Chapter 2).

Correlation between metric traits

The relationship between the metric traits was examined next (Table 5.11). The correlations between the four size variables were calculated for yearling and adult males and females separately due to the age related change in plumage dimensions and excluding data collected between June and September when weight variation is extreme and unpredictable. Wing and tail length were consistently the most highly correlated characters usually followed by weight and tarsus. Most were positively correlated and thus each reflected overall size to a large extent. However, tail length variation was largely independent of weight and tarsus length. The direction of change of the correlation coefficients between yearlings and adult were similar for both sexes other than for wing with weight. No major changes between relationships were observed other than the declining correlation between plumage length and skeletal size estimated from tarsus measurements

Assortative mating

Burke found weak evidence for assortative mating at the Sutton Bonington site for weight and tail length but not at Brackenhurst. Data from 1986 to 1988 showed that the Sparrows had mated assortatively for tail length (Table 5.12). The pooled data may have been biased by the progressive though non significant increase in plumage length during this period. However, 1987 and 1988 pairs alone gave significant results.

Table 5.11**Product moment correlations between metrics****Yearling males (N = 149)****Yearling females (N = 101)**

	T2	Wing	Tail	T2	Wing	Tail
Wing	0.256 ***			0.278 ***		
Tail	0.054	0.534 ****		-0.005	0.500 ****	
Weight	0.407 ****	0.250 ***	-0.017	0.277 ***	0.295 ***	0.104

* = P < 0.05, ** = P < 0.01, *** = P < 0.005, **** = P < 0.001

Full adult males (N = 130)**Full adult females (N = 84)**

	T2	Wing	Tail	T2	Wing	Tail
Wing	0.164			0.150		
Tail	-0.095	0.572 ****		-0.121	0.541 ****	
Weight	0.481 ****	0.297 ***	0.017	0.420 ****	0.082	0.135

* = P < 0.05, ** = P < 0.01, *** = P < 0.005, **** = P < 0.001

Correlations were calculated using the mean of all measurements of an individual within each age range, but excluding data collected between June and September when weight fluctuates significantly.

Table 5.12**Product moment correlation between metrics of mated pairs**

Year	Age	T2	Weight	N	Wing	Tail	N
1985	-0.216	0.475	0.324	18	-	-	0
1986	0.133	0.002	0.149	41	0.229	-0.013	38
1987	0.112	0.226	0.045	38	-0.041	0.441 *	27
1988	-0.047	-0.146	0.494	13	0.206	0.674 *	7

The values used in the correlation analysis were the mean of post-fledge T2 measurements, mean of post-juvenile weight, and mean plumage recorded in the year of breeding. Birds of known and estimated age were pooled. (* = $P < 0.05$)

Since plumage length is known to be correlated with age, this was also included in the analysis. Although a positive association exists between the ages of members of a pair, it was not significant, and thus was unlikely to have resulted in the correlation between tail lengths. Yearlings fledged from early broods grow longer plumages and may pair in the autumn whilst the short-tailed fledglings from later broods pair in the spring and summer and in so doing might appear to show positive assortment within the yearling class. Pairs are usually faithful between years thereby increasing the number of (long-tailed) full adult pairs above expectation. However, no relationship was found between the fledging date of the last brood, which is followed shortly by the onset of the post-nuptial moult, and tail length in the next year which might increase the correlation between members of faithful pairs. Neither are early fledging, long-tailed yearlings more likely to pair with full adults (or each other) rather than late fledging, short-tailed yearlings. Therefore, the most likely mechanisms which do not involve mate choice cannot explain a significant proportion of the correlation between mates.

Genetic components of metric variation

The previous sections have described the range of variation and some of the factors which influence the four metric traits measured at Brackenhurst. Previous studies have demonstrated an important heritable genetic component in size variation of these traits in other species (reviewed by Boag and van Noordwijk, 1987). The following sections will describe how the techniques of quantitative genetics can be used to partition variance into genetic and environmental components (Falconer, 1981).

The total phenotypic variance (V_p) in a population is the sum of the following components:

$$V_p = V_G + V_E$$

where V_G is the genetic and V_E the environmental variances. Each can be subdivided as follows:

$$V_G = V_A + V_D + V_I$$

and

$$V_E = V_{Eg} + V_{Es}$$

Here V_A is the additive genetic variance which is inherited directly from the parents. V_D , the dominance variance and V_I , the interaction variance, represent genetically determined variation which is not simply transmitted from parent to offspring due to new dominance and epistatic combinations formed in the offspring through recombination of the parental alleles and genes. The residual environmental variance is assigned to V_E which is subdivided into two components. These are V_{Es} , the special environmental variance defined by Falconer (1981) as the “within-individual variance arising from temporary or localized circumstances”, which will include measurement error, changes in weight between days and fluctuation in plumage length due to abrasion and moult etc. The general environmental variance, V_{Eg} is defined as “arising from permanent or non localized circumstances” such as variation in adult tarsus length influenced by food availability in the nest.

Of the genotypic variance, the most interesting and usually the largest component is V_A . Being the only one which is heritable in the strict sense it determines the magnitude of the response to selection on a quantitative trait. Heritability (h^2) is equal to the ratio V_A/V_P and is thus clearly dependent on the amount of environmental variation (V_E). In order to demonstrate significant heritabilities the special environment variance must be minimized. This requires careful, accurate measurements and the selection of homogeneous data subsets which exclude as much temporal variation as possible.

Repeatability measures the fraction of phenotypic variance resulting from “permanent, or non-localized differences between individuals both genetic and environmental” and is formally defined as:

$$r = \frac{V_G + V_{Eg}}{V_G + V_{Eg} + V_{Es}} = \frac{V_G + V_{Eg}}{V_P}$$

Hence, repeatability sets the upper limit to the ratio V_A/V_P and shows how a large V_E can mask the importance of additive genetic variance (Falconer, 1981).

Heritability analysis uses multiple measurements from related individuals to quantify the effect of shared genes on phenotypic similarity. In the absence of environmental variation, offspring will equal the mean size of their parents since they inherit half of the genes determining additive size variation from each. If, however, there is significant environmental variation the mean offspring values will diverge away from the mid-parent values towards the population mean. The magnitude of the drift being proportional to the relative sizes of V_G and V_E .

The simplest method for estimating heritability is to regress the mid-offspring values of broods on their mid-parent values. The slope of the resulting regression line equals the heritability. Alternatively, offspring can be regressed on single parent values and the slope doubled (since offspring share only half their genes with each parent). The former method is preferable as it is both more accurate and unaffected by assortative mating. Single parent regressions assume the other parent is randomly drawn from the population, hence heritabilities estimated using this method are inflated by positive assortative mating.

In common with all regression based analyses it is important to ensure that the measurements are normally distributed and have no outliers. In previous studies which

lacked genetic confirmation of parentage, the incorrect assignment of a few parents due to EPF or egg dumping could have had a large effect on the slope. The Brackenhurst data set comprising more than 350 offspring and their confirmed parents is ideal for such an analysis.

Heritability of tarsus length

The tarsi of nestling House Sparrows have attained their adult size by the time of bleeding, ringing and measuring at 11 days of age. Therefore this metric provides the largest sample of parent offspring trios. An initial plot of mid-parent value against mid-offspring (the mean for each brood) revealed a considerable degree of scatter in the data. Possible correlates with V_E were tested by multiple analysis of variance. These include year, date of clutch initiation, brood size at incubation, hatching and fledging, proportion of hatchlings surviving to fledge, presence of EPOs, parental age and a variety of interactions between these factors.

Parental age, the occurrence of cuckoldry and clutch size had no effect. Neither year nor date of clutch initiation alone was important but date within year was. Being an early or late brood had no effect *per se* but in particular years birds varied significantly in size between months. Presumably unpredictable interactions between weather and food supply were responsible.

Data from the three years 1986-1988 were analysed separately to allow for temporal variation. The mean size of the nestlings from each brood was regressed against each attendant with day, number of hatchlings and proportion of hatchlings surviving to fledge as additional predictor variables. The analysis was carried out

twice, firstly including EPOs and then without. Finally the mid-parent values were regressed on the mid-WPO value for each brood.

In 1986 none of the parent offspring regressions yielded a significant slope though all were positive. However, the proportion of nestlings surviving to fledge was positively correlated with mid-nestling tarsus length ($t = 2.57$, $F_{1,39} = 7.91$, $P < 0.01$). In 1987 temporal variation was more important, larger nestlings being found in the later broods. In this year the regression on females was not significant whereas male tarsus length was strongly correlated with offspring size. In 1988 the same pattern was repeated though date was not a significant factor. In both 1987 and 1988 mid-parent regressions gave significant slopes.

These findings show that environmentally induced variation fluctuates in importance. The small size of nestlings from broods with high mortality may reflect a poor food supply to these broods because most nestling mortality is the result of starvation (Seel, 1970). The higher heritability calculated from the male/nestling analysis is surprising. It was expected that mother/offspring slopes would be greater than those of the male against all nestlings, because of the inclusion of EPOs, and similar to that of fathers on WPOs. The exclusion of EPOs from the analysis in each case increased the proportion of nestling variance explained by male size, but the improvement was not significant. The closer relationship between nestlings and their male attendant may be the result of a "paternal" effect whereby large males raise large nestlings for non-genetic reasons, for example long limbed males might provide more parental care. What basis this would have is unknown, but evidence of a positive correlation between bib size and parental care has been found (Møller, 1990).

Table 5.13a

Heritability of tarsus length

Year	Comparison	Regression coefficient	S.E.	t	Analysis of Variance				Residual d.f.
					Attendant size	Date	Number Hatch	Proportion Fledge	
1986	Female/all nestlings	0.177	0.167	1.06	1.12	1.50	2.35	8.44 **	40
	Female/WPO	0.171	0.171	1.00	1.01	1.66	3.75	7.69 **	39
	Male/all nestlings	0.162	0.174	0.93	0.87	1.49	2.34	8.39 **	40
	Male/WPO	0.207	0.176	1.18	1.39	1.67	3.78	7.76 **	39
	Mid-Parent/WPO	0.340	0.229	1.48	2.19	1.71	3.86	7.91 **	39
1987	Female/all nestlings	0.052	0.087	0.60	0.36	5.37 *	0.06	0.94	46
	Female/WPO	0.109	0.085	1.24	1.53	4.45 *	0.78	0.64	40
	Male/all nestlings	0.296	0.079	3.73	13.9 ***	6.94 *	0.08	1.21	46
	Male/WPO	0.348	0.074	4.71	22.2 ***	6.66 *	1.17	0.96	40
	Mid-Parent/WPO	0.375	0.101	3.70	13.7 ***	5.75 *	1.01	0.83	40
1988	Female/all nestlings	0.389	0.211	1.85	3.41	1.07	0.11	0.32	13
	Female/WPO	0.511	0.256	2.00	4.01	0.02	0.59	0.21	9
	Male/all nestlings	0.652	0.299	2.18	4.75	1.16	0.12	0.34	13
	Male/WPO	0.690	0.293	2.35	5.55 *	0.02	0.66	0.23	9
	Mid-Parent/WPO	1.405	0.226	6.22	38.7 ***	0.07	2.18	0.75	9

Heritability = regression coefficient for mid-parent comparisons and twice the slope for all other comparisons

* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.005$, **** = $P < 0.001$

Table 5.13b

Heritability of weight

Year	Comparison	Regression coefficient	S.E.	t	Analysis of Variance				Residual d.f.
					Attendant size	Date	Number Hatch	Proportion Fledge	
1986	Female/all nestlings	0.202	0.246	0.82	0.68	0.62	7.08 *	1.12	24
	Female/WPO	0.139	0.250	0.55	0.31	0.51	6.67 *	1.73	23
	Male/all nestlings	0.562	0.206	2.72	7.41 *	0.78	9.01 **	1.42	24
	Male/WPO	0.557	0.207	2.70	7.26 *	0.66	8.67 **	2.25	23
	Mid-Parent/WPO	0.612	0.292	2.10	4.41 *	0.60	7.85 **	2.04	23
1987	Female/all nestlings	0.351	0.223	1.57	2.47	1.47	0.03	0.07	34
	Female/WPO	0.361	0.264	1.37	1.87	0.23	0.28	0.07	30
	Male/all nestlings	0.241	0.175	1.38	1.90	1.44	0.03	0.07	34
	Male/WPO	0.295	0.197	1.50	2.24	0.23	0.28	0.07	30
	Mid-Parent/WPO	0.860	0.345	2.49	6.20 *	0.26	0.32	0.08	30
1988	Female/all nestlings	0.280	1.120	0.25	0.06	0.38	0.91	3.13	8
	Female/WPO	0.570	1.420	0.40	0.16	0.34	2.09	2.21	6
	Male/all nestlings	0.740	0.576	1.28	1.65	0.46	1.09	3.75	8
	Male/WPO	0.607	0.697	0.87	0.76	0.37	2.29	2.42	6
	Mid-Parent/WPO	0.800	1.020	0.78	0.61	0.36	2.24	2.37	6

Heritability = regression coefficient for mid-parent comparisons and twice the slope for all other comparisons

* = $P < 0.05$, ** = $P < 0.01$

Weight

Nestlings were ringed at the peak of the weight growth curve just prior to a slight decline when fat reserves which serve as insulation are metabolized. Despite the fact that full adult weight is rarely achieved at this stage, nestling weight is still highly correlated with over-winter weight ($N = 49$, $r = 0.578$, $P < 0.001$) and was therefore used to predict heritability as above.

In 1986 the number of hatchlings was negatively correlated with weight at ringing, probably as a consequence of intra-brood competition for limited food resources. The regression on male weight in this year was significant but no improvement was gained by excluding EPOs. No significant correlates with nestling weight were found in 1987 or 1988 other than mid-parent size in the latter year. Similar estimates of heritability from mid-parent regressions in all three years suggest that additive genetic variance can account for 60-80% of nestling weight variance but environmental variation results in large standard errors for the estimates.

Plumage length

The problems associated with estimating heritabilities of wing and tail length are compounded by the variation between age classes which necessitate the comparison of parents and offspring at the same age. Unfortunately, only seven confirmed parent/offspring trios were available when comparing yearling plumages and four of these involved full sibs which were pooled to give a mid-offspring value. All measurements were collected between 1986 and 1989. The mid-parent analysis gave a tail length heritability estimate of $0.626 (\pm 0.107, t = 5.87, P < 0.05)$ but no relationship was found for wing length ($h^2 = 0.942 \pm 0.843, t = 1.12, N.S.$).

However, the accuracy of these estimates is doubtful because of the small sample sizes involved.

Significant relationships were found between mid-offspring and mid-parent size for three of the metric traits measured at Brackenhurst. Clearly additive genetic variance accounts for a substantial proportion of size variation. Nestling tarsus length and weight are known to be closely correlated ($r = 0.434$, $P < 0.001$, $N = 409$) and thus some of the heritability of these two traits will reflect a shared set of pleiotropic body size genes. The demonstrable heritability of tail length is more surprising given the amount of age related variation and the small sample size. Heritability of plumage traits are seldom assessed for these reasons, but a large heritable component of wing length variation has been successfully shown in Indigo Buntings (Payne and Payne, 1989). The failure to demonstrate heritability of wing length in House Sparrows is probably a result of the small sample size and environmental variation rather than a lack of genetic variation.

The inconsistency of heritability estimates of tarsus length in this study may be due to a variety of factors. Repeatabilities of tarsus length were somewhat lower than in studies of some other species. This is partly due to measurement error, e.g. occasional measurements must have been taken at the wrong scute when they had unusual boundaries, and to a lower coefficient of variation in Sparrows compared with, for example, *Geospiza fortis* (Grant, 1986) which has a much greater size range. More importantly the limited food supply which results in 59% of broods suffering mortality from starvation must affect nestling size.

As a result of the large standard errors associated with the heritability estimates, an analysis of EPF rate using the methods of Alatalo and Lundberg (1986) could not be attempted. The species in which this has been successfully used (e.g. Pied Flycatchers and Swallows) seldom suffer starvation. Data from the Great Tit reveals significant

heritabilities for early broods and broods with high survivorship, whilst late broods and those suffering mortality through starvation had a much greater range of nestling weights and no detectable relationship with parental size (Boag and van Noordwijk, 1987). Therefore the reliability of this method is dependent on an adequate food supply and large sample sizes.

Summary

Quantitative variation in weight, wing, tail and tarsus length was examined at the Brackenhurst population between October 1986 and February 1989. All four traits were sexually dimorphic but variation was also found between cohorts. Plumage length increases significantly between the yearling and second adult plumages. The shorter plumage of yearlings is to some extent related to their fledging date which in turn affects the age at which the post-juvenile moult is entered. Dietary restriction at this time might have an important influence on yearling size.

Food availability during nestling growth also has an effect on body size. Broods with no mortality produce longer limbed fledglings in some years whilst intra-brood competition can affect an individual's weight as fledging is approached. The smallest nestlings in most years die soon after fledging leading to a significant increase in tarsus length between the nestling and juvenile age classes. Those which survive the transition to independence then determine the size of yearlings in the next breeding season. A heritable component to three of the traits can be demonstrated, Failure to do so for wing length probably resulted from the small sample size. However, the confidence limits placed on the estimates were very wide. An examination of these traits in a year when invertebrates are particularly abundant may produce more reliable estimates.

Evidence of assortative mating for tail length was obtained in two years (1987 and 1988). Similar evidence was reported by Burke at Sutton Bonington. The similarity between mates was not significantly dependent on age related changes. The poor correlation between tail length and other measures of body size suggests that tail length itself may play some role in mate choice.

CHAPTER 6

FUTURE PROSPECTS

A multidisciplinary approach to the biology of animal populations is becoming commonplace (e.g. Grant, 1986; Woolfenden and Fitzpatrick, 1984; Newton, 1986). Long term ringing studies in particular have allowed the reconstruction of life history traits for individual birds which can be related to differences in their behaviour, habitat and morphology. A better knowledge of all aspects of an individual's biology would help us to understand the observed differences in individual fitness. For example, Grant (1986) has demonstrated how body size and beak morphology of Darwin's Finches are influenced by both the environment and additive genetic variance. These, having been determined in the nest then influence a male's chances of obtaining a high quality territory which can, in turn, influence the morphology of its offspring. Size also plays a major role in the feeding ecology of each bird determining which food items it can handle and thus whether it survives periods of drought and famine.. Size may also play a role in mate choice. As more species are studied in the depth that Grant and his co-workers have achieved, our understanding of population biology will increase dramatically and general trends and rules may appear.

One of the primary areas of interest is the estimation of lifetime reproductive success (LRS). This is currently the best measure of fitness available. It is usually estimated by one of two correlated values, the number of fledglings or recruits to future breeding populations produced during an individual's lifetime (Newton, 1989). Many "cross-sectional" studies have attempted to identify the factors which affect the success of individuals using data from a single breeding season, but the variance between the lifetime reproductive success of individuals is a far more important and realistic measure of an individuals fitness. In a compilation of studies of birds (Newton, 1989) Newton emphasized that lifespan has a major influence accounting for 30 - 86% of variance in fledgling production. The massive mortality involved in maintaining a stable population size combined with lifespan variation, differential mating success and

pure luck results in a huge variance in the number of offspring produced by each fledgling, e.g. 3% of Blue Tit fledglings contribute 50% of recruits in the next generation.

In none of the studies in Newton's review is the extent of mixed reproductive strategies estimated and yet studies based on plumage markers, heritability of metric traits, enzyme polymorphisms and RFLPs have reported the common occurrence of EPC and intra-specific brood parasitism resulting in 1 - 42% of nestlings being the biological offspring of individuals other than their attendants (Burke *et al.*, 1989 and Westneat, 1987a respectively). It has previously been assumed that the mean and variance of LRS in monogamous birds would not vary greatly unless the sexes differed in survival. However, the effect of EPC is to increase the variance in male reproductive success. Should older males be more successful in gaining EPCs the variance will increase dramatically as individuals which live long enough to raise many of their own offspring will also have the opportunity to sire many through EPFs.

The importance of alternatives to monogamy cannot be over-emphasized in the affect they may have on the population structure, because the major limitation on male reproductive success is the number of females that can be fertilized. If females display a preference for particular males both as mates and as partners in EPC, then the contribution of these individuals to the future generation will greatly outweigh that of the victims of their cuckoldry. Female preferences for particular males have been clearly demonstrated in the monogamous Swallow and House Sparrow (Møller, 1988b, 1989a and 1990).

More information must be obtained on the costs and benefits of pursuing mixed reproductive strategies. The benefits to the male seem overwhelming but does indulgence in EPC affect a male's confidence of paternity of the brood raised by his own mate? The possible benefits a female might gain through EPC range from superior

genes for her offspring, parental care from the males with which she has copulated and an insurance against infertility, but the risks include reduced aid from the cuckolded mate, desertion and physical injury during forced extra-pair copulations (McKinney *et al.*, 1984). The extent to which females cooperate in EPCs is unclear because submission to an attempted EPC may not reflect willingness to mate but avoidance of physical abuse. Detailed observations of the reproductive behaviour of individual males and females must be combined with a means of determining the genetic outcome in order to clarify this problem.

Observations made during this study, combined with the literature on House Sparrow ecology, behaviour and reproductive success, demonstrate that much work remains to be done to identify the causes and consequences of LRS variation in this species.

Nestling mortality is high at 40% and 80% of fledglings die before they reach their first breeding season, during which some fail to attract a mate whilst others attempt to raise broods but fail. Adults suffer a mortality rate of ~50%/year. Thus, few birds ever succeed in raising a brood whilst the longest survivors can reach 8 years of age and may produce up to 35 fledglings (pers. obs.). At Brackenhurst simply counting fledglings may provide the best estimate of female LRS. However we have evidence that cuckoldry can greatly affect the number of offspring sired by each male. Some individuals never raised a nestling of their own despite successfully fledging more than one brood whilst others sired at least 5 offspring through cuckoldry. The occurrence of natural nests, unringed birds, incomplete blood sampling and the limited time span of the study meant that the costs in terms of the number of EPOs raised by each male could not be measured. In the future it is hoped that some of the benefits in number of EPOs sired might be quantified as well.

To achieve the latter will require the development of single locus minisatellite probes (Wong *et al.*, 1986 and 1987; Gyllenstein *et al.*, 1990; Burke, 1989). These probes detect the alleles of a particular hypervariable locus revealing a one or two banded phenotype following the procedures outlined in Fig. 4.1. The extreme variability of some loci will allow the assignment of paternity on the basis of possession of rare alleles. Once the allele frequencies have been determined for each locus the combined probability of parental inclusion can be calculated. The development of internal mapping techniques (Jeffreys *et al.*, 1990) has shown that variation between alleles at a single locus is sufficient for individual identification in outbred human populations. The application of techniques of this sophistication, though it may be many years in the future, could revolutionize demographic studies just as multilocus fingerprinting is starting to do.

The fingerprinting protocols used in this study are capable of detecting all cases of EPF by unrelated males. However, a proportion of nestlings sired by close relatives of the attendant male might have been missed. Though an unlikely occurrence, this weakness arose through the choice of Hae III as the enzyme for restriction digests. This enzyme gives relatively fewer informative bands compared with Alu I because of the rarity of probe specific fragments detected by pSPT 18.15. After the initial choice of Hae III, time and monetary restrictions pre-determined that the entire survey would be completed with this enzyme. However, the choice may prove to be fortuitous in that a large proportion of the fragments detected by pSPT 19.6 must be located on the Z chromosome. Evidence of a high frequency of allelism in males suggest that these alleles are consistently large and therefore provide an opportunity to produce single locus probes which could be used to rescreen the blots used in the fingerprinting survey. If the Z-linked loci are hypervariable, as the evidence suggests, then female nestlings could be identified with high probability by their possession of the maternal allele.

The increased sensitivity of single locus probes (SLPs) derives from their greater homology with the target DNA compared with the multilocus probes of human origin. Using SLPs a visible signal can be obtained from smaller quantities of genomic DNA than are required for standard fingerprints. This will enable the routine examination of young nestlings and fresh corpses. The increased sample sizes could be used to examine the sex ratio soon after hatching and provide a more complete data set for the analysis of EPO distribution.

An understanding of the role played by male fertility levels in selecting for female cooperation in EPCs and in determining the outcome of sperm competition requires a detailed knowledge of the breeding status and reproductive behaviour of both the cuckolding and cuckolded males. This demands focal observations of particular individuals and the use of single locus probes to rapidly screen the population for potential biological parents.

The observed correlation between infertility and cuckoldry has been strengthened by examination of the seven eggs which failed to hatch in the 39 fingerprinted broods sampled in 1989. Four of the eggs were found to be infertile. Using this data a contingency table categorizing broods by the presence of EPOs and infertile eggs was constructed. Four of the 14 EPO containing broods had a single infertile egg but none was found amongst the 25 legitimate broods. This result provides significant confirmation that cuckoldry is most likely to succeed when the pair produces infertile eggs ($P = 0.012$, Fishers Exact Probability Test).

The increasing body of data relating bib size to dominance in social interactions and mating success should be tested in a population where parentage is monitored by DNA fingerprinting. Observations of pair formation could be used to correlate the relative proportion of WPOs and EPOs within a brood with the date when pairs form. Early pairing might offer more opportunities for mate choice if the pool of available

mates is larger. If females are frustrated in their mate choice by the limited availability of unpaired males they might be more likely to seek EPCs from males with the preferred phenotypes, e.g. large bibs. However, strong evidence of mate choice in one population does not guarantee its occurrence in another. Several behaviours observed at high frequency in other Sparrow populations were not recorded at Brackenhurst, e.g. helping behaviour (Sappington, 1975), infanticide (Veiga, 1990) and egg dumping. The absence of the latter was almost certainly due to the abundance of suitable nesting sites, whereas the conditions which affect the occurrence of the other behaviours is unknown.

The application of DNA fingerprinting to the Brackenhurst House Sparrow population has revealed the necessity of detecting all extra-pair fertilizations if we are to understand the factors which influence lifetime reproductive success and the genetic structure of populations. In particular, the role of plumage traits in mate selection, the importance of natural variation in fertility and the costs and benefits of mixed reproductive strategies are promising avenues for future investigation in this species. The accumulated wealth of one hundred years research clearly contradicts Alfred Newton's entry for the House Sparrow in "*A Dictionary of Birds*" (1896).

“Far too well known to need any description
of its appearance or habits.”

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APPENDIX 1

Electromorphs and allele frequencies for each locus subdivided by age, sex and year. The pullus class includes all nestlings and the juveniles were bled whilst retaining their immature plumage. All birds in the male and female classes were adults. Individuals which might have been bled prior to their first winter but which had not retained any juvenile plumage were excluded from the analysis.

G_{HW} = goodness of fit G-tests for agreement with Hardy Weinberg ratios.

IDH

Female	AA	AB	BB	N	A	B	G_{HW}
1980	17	16	2	35	.714	.286	0.53
1981	16	14	4	34	.676	.324	0.12
1982	18	19	9	46	.598	.402	0.91
1983	16	22	3	41	.659	.341	1.60
1984	7	5	2	14	.679	.321	0.45
1985	40	28	7	75	.720	.280	0.40
1986	23	26	3	52	.692	.308	1.66
1987	18	9	3	30	.750	.250	1.13
1988	10	9	0	19	.763	.237	1.83
Total	165	148	33	346	.691	.309	0.00
H.W.	165.1	147.8	33.1				

Male	AA	AB	BB	N	A	B	G_{HW}
1980	20	7	3	30	.783	.217	2.61
1981	10	21	3	34	.603	.397	2.97
1982	17	7	1	25	.820	.180	0.06
1983	21	19	0	40	.763	.238	3.88
1984	3	2	1	6	.667	.333	0.37
1985	56	38	12	106	.708	.292	1.84
1986	29	25	6	60	.692	.308	0.03
1987	13	12	1	26	.731	.269	0.85
1988	20	13	3	36	.736	.264	0.17
Total	189	144	30	363	.719	.281	0.12
H.W.	187.7	146.7	28.6				

IDH

P	AA	AB	BB	N	A	B	GHW
1980	10	9	0	19	.763	.237	1.83
1981	63	50	7	120	.733	.267	0.53
1982	75	63	32	170	.626	.374	7.31
1983	70	66	26	162	.636	.364	2.32
1984	83	42	19	144	.722	.278	10.18
1985	65	46	14	125	.704	.296	1.67
1986	93	91	18	202	.686	.314	0.41
1987	72	76	25	173	.636	.364	0.45
1988	30	27	13	70	.621	.379	2.26
Total	561	470	154	1185	.672	.328	11.84
H.W.	534.7	522.6	127.7				

J	AA	AB	BB	N	A	B	GHW
1980	15	17	1	33	.712	.288	2.47
1981	5	8	1	14	.643	.357	0.88
1982	13	4	0	17	.882	.118	0.30
1983	-	-	-	-	-	-	-
1984	-	-	-	-	-	-	-
1985	3	2	1	6	.667	.333	0.37
1986	20	22	4	46	.674	.326	0.36
1987	15	7	3	25	.740	.260	1.73
1988	21	14	3	38	.737	.263	0.09
Total	92	74	13	179	.670	.330	0.13
H.W.	93.0	72.0	14.0				

6PGD

Female	AA	AB	BB	N	A	B	G _{HW}
1980	-	3	32	35	.043	.957	0.07
1981	-	3	31	34	.044	.956	0.07
1982	-	2	44	46	.022	.978	0.02
1983	-	2	39	41	.024	.976	0.03
1984	-	1	14	15	.033	.967	0.02
1985	1	10	66	77	.078	.922	0.57
1986	-	8	45	53	.075	.925	0.35
1987	-	2	27	29	.034	.966	0.04
1988	-	1	18	19	.026	.974	0.01
Total	1	32	316	349	.049	.951	0.04
H.W.	0.8	32.4	315.8				

Male	AA	AB	BB	N	A	B	G _{HW}
1980	-	1	30	31	.016	.984	0.01
1981	-	4	30	34	.059	.941	0.13
1982	-	1	24	25	.020	.980	0.01
1983	-	2	38	40	.025	.975	0.03
1984	-	-	6	6	.000	1.000	-
1985	-	6	100	106	.028	.972	0.09
1986	-	1	60	61	.008	.992	0.00
1987	-	1	25	26	.019	.981	0.01
1988	1	1	34	36	.042	.958	5.33
Total	1	17	347	365	.026	.974	1.42
H.W.	0.3	18.5	346.2				

6PGD

P	AA	AB	BB	N	A	B	G _{HW}
1980	-	0	19	19	.000	1.000	-
1981	-	6	114	120	.025	.975	0.08
1982	-	12	158	170	.035	.965	0.23
1983	-	2	163	165	.006	.994	0.01
1984	-	3	141	144	.010	.990	0.02
1985	-	5	120	125	.020	.980	0.05
1986	-	20	175	195	.051	.949	0.57
1987	-	18	154	172	.052	.948	0.52
1988	-	1	59	60	.008	.992	0.00
Total	0	67	1103	1170	.029	.971	1.02
H.W.	1.0	65.0	1104.0				

J	AA	AB	BB	N	A	B	G _{HW}
1980	-	3	30	33	.045	.955	0.07
1981	-	-	14	14	0.00	1.000	-
1982	-	-	17	17	0.00	1.000	-
1983	-	-	-	-	-	-	-
1984	-	-	-	-	-	-	-
1985	-	1	5	6	.083	.917	0.05
1986	-	3	43	46	.033	.967	0.05
1987	-	3	22	25	.060	.940	0.10
1988	-	5	32	37	.068	.932	0.19
Total	0	15	163	178	.042	.958	0.34
H.W.	0.3	14.4	163.3				

PEPD2

Female	AA	AB	BB	N	A	B	G_{HW}
1980	-	2	33	35	.029	.971	0.03
1981	-	2	32	34	.029	.971	0.03
1982	-	5	41	46	.054	.946	0.15
1983	-	3	38	41	.037	.963	0.06
1984	-	1	14	15	.033	.967	0.02
1985	-	8	69	77	.052	.948	0.23
1986	1	2	50	53	.038	.962	4.42
1987	-	6	24	30	.100	.900	0.37
1988	-	1	17	18	.046	.954	0.01
Total	1	30	318	349	-	-	.01
H.W.	0.7	30.6	317.7				

Male	AA	AB	BB	N	A	B	G_{HW}
1980	-	6	25	31	.097	.903	0.36
1981	-	2	32	34	.029	.971	0.03
1982	-	2	23	25	.040	.960	0.04
1983	1	3	36	40	.063	.938	2.74
1984	-	-	6	6	.000	1.000	-
1985	1	10	95	106	.057	.943	1.00
1986	-	5	56	61	.041	.959	0.11
1987	-	3	23	26	.058	.942	0.10
1988	-	5	30	35	.071	.929	0.21
Total	2	36	326	364	.055	.945	0.68
H.W.	1.1	37.8	325.1				

PEPD2

P	AA	AB	BB	N	A	B	G _{HW}
1980	-	1	18	19	.026	.974	0.01
1981	-	13	108	121	.054	.946	0.39
1982	-	19	151	170	.056	.944	0.60
1983	3	11	151	165	.052	.948	8.30
1984	-	6	138	144	.021	.979	0.07
1985	-	18	107	125	.072	.928	0.75
1986	-	14	188	202	.035	.965	0.26
1987	1	14	158	173	.046	.954	0.84
1988	-	5	59	64	.039	.961	0.11
Total	4	101	1078	1183	.046	.954	0.84
H.W.	2.5	104.0	1076.5				

J	AA	AB	BB	N	A	B	G _{HW}
1980	-	2	31	33	.030	.970	0.03
1981	-	-	14	14	.000	1.000	-
1982	-	1	16	17	.029	.971	0.02
1983	-	-	-	-	-	-	-
1984	-	-	-	-	-	-	-
1985	-	-	6	6	.000	1.000	-
1986	1	2	42	45	.044	.956	4.09
1987	-	4	21	25	.080	.920	0.19
1988	-	3	34	37	.041	.959	0.07
Total	1	12	164	177	.043	.957	1.29
H.W.	0.3	13.4	163.3				

GP

F	AA	AB	BB	AC	BC	CC	N	A	B	C	G _{HW}
1980	2	6	22	-	1	-	31	.161	.823	.016	2.63
1981	2	7	18	1	4	-	32	.188	.734	.078	1.31
1982	1	15	29	-	1	-	46	.185	.804	.011	0.59
1983	3	12	21	-	1	-	37	.243	.743	.014	0.80
1984	2	6	7	-	-	-	15	.333	.667	.000	0.15
1985	3	28	43	-	2	-	76	.224	.763	.013	0.98
1986	3	20	28	-	1	1	53	.245	.726	.028	23.05
1987	-	5	24	1	-	-	30	.100	.883	.017	9.32
1988	-	6	12	-	1	-	19	.158	.816	.026	0.97
Total	16	105	204	2	11	1	339	.205	.773	.022	2.66
H.W	14.2	107.4	202.5	3.1	11.6	0.2					

M	AA	AB	BB	AC	BC	CC	N	A	B	C	G _{HW}
1980	1	6	20	-	1	-	28	.143	.839	.018	0.58
1981	2	8	19	-	4	1	34	.176	.735	.088	4.07
1982	-	10	13	-	1	-	24	.208	.771	.021	2.12
1983	3	10	19	-	2	-	34	.235	.735	.029	1.64
1984	1	2	3	-	-	-	6	.333	.667	.000	0.37
1985	10	32	59	2	3	-	106	.252	.715	.023	3.50
1986	1	16	44	-	-	-	61	.148	.852	.000	0.12
1987	-	10	14	1	1	-	26	.212	.750	.038	2.55
1988	3	13	19	-	-	-	35	.271	.729	.000	0.13
Total	21	107	210	3	12	1	354	.215	.761	.024	3.80
H.W	16.3	115.7	205.2	3.7	12.9	0.2					

GP

P	AA	AB	BB	AC	BC	CC	N	A	B	C	G _{HW}
1980	1	3	12	2	1	-	19	.184	.737	.079	5.95
1981	10	38	59	5	9	-	121	.260	.682	.058	2.04
1982	9	66	86	4	5	-	170	.259	.715	.026	2.30
1983	7	70	83	2	1	-	163	.264	.727	.009	5.17
1984	15	49	70	2	7	-	143	.283	.685	.031	2.34
1985	9	34	81	-	-	-	124	.210	.790	.000	3.39
1986	13	58	124	4	3	-	202	.218	.765	.017	7.91
1987		50	109	6	6	-	174	.178	.787	.034	9.64
1988	4	14	51	-	-	-	69	.159	.841	.000	3.40
Total	71	382	675	25	32	0	1185	.232	.744	.024	16.91
H.W	63.6	408.6	656.5	13.2	42.4	0.7					

J	AA	AB	BB	AC	BC	CC	N	A	B	C	G _{HW}
1980	6	10	16	1	-	-	33	.348	.636	.015	4.84
1981	2	1	10	-	1	-	14	.179	.786	.036	8.06
1982	2	7	5	1	2	-	17	.353	.559	.088	0.24
1983	-	-	-	-	-	-	-	-	-	-	-
1984	-	-	-	-	-	-	-	-	-	-	-
1985	-	1	5	-	-	-	6	.083	.917	.000	0.05
1986	1	12	31	1	-	-	45	.167	.822	.011	5.06
1987	1	5	17	-	2	-	25	.140	.820	.040	1.03
1988	2	7	24	-	1	-	34	.162	.824	.015	2.10
Total	14	43	108	3	6	0	174	.212	.762	.026	9.28
H.W	7.9	56.3	100.9	1.9	6.9	0.1					

PEPD3

F	AB	BB	BC	BD	BX	CD	DD	N	A	B	C	D	X	G _{HW}
1980	-	29	-	4	-	-	-	33	-	.939	-	.061	-	0.14
1981	2	25	1	3	-	-	-	31	.032	.904	.016	.048	-	0.36
1982	1	37	-	3	-	-	-	41	.012	.951	-	.037	-	0.11
1983	-	36	-	2	-	-	-	38	-	.974	-	.026	-	0.03
1984	-	9	2	3	-	-	-	14	-	.822	.071	.107	-	0.66
1985	1	54	2	10	-	-	1	68	.007	.890	.015	.088	-	0.04
1986	-	43	-	6	-	-	-	49	-	.939	-	.061	-	0.21
1987	1	23	-	-	-	-	-	24	.021	.979	-	-	-	0.01
1988	-	15	-	1	-	-	-	16	-	.969	-	.031	-	0.02
Total	5	271	5	32	-	-	1	314	.008	.930	.008	.054	-	0.25
H.W	4.7	271.6	4.7	31.5	-	-	0.9							

M	AB	BB	BC	BD	BX	CD	DD	N	A	B	C	D	X	G _{HW}
1980	-	23	-	4	-	-	-	27	-	.926	-	.074	-	0.17
1981	1	27	-	4	-	-	-	32	.016	.921	-	.063	-	0.23
1982	1	20	-	1	-	-	-	22	.023	.954	-	.023	-	0.05
1983	1	27	3	6	-	-	-	36	.014	.861	.042	.083	-	0.90
1984	-	3	1	1	-	1	-	6	-	.666	.167	.167	-	0.37
1985	4	78	5	8	-	-	1	96	.021	.901	.026	.052	-	0.00
1986	2	41	2	10	1	-	-	56	.018	.866	.018	.089	.009	1.34
1987	-	18	1	3	-	-	-	22	-	.909	.023	.068	-	0.22
1988	-	23	2	4	-	-	-	29	-	.897	.034	.069	-	0.39
Total	9	260	14	41	1	1	1	327	.014	.891	.023	.070	.002	1.07
H.W	8.2	259.6	13.4	40.8	1.2	1.1	1.6							

PEPD3

P	AB	BB	BC	BD	BX	CC	DD	N	A	B	C	D	X	GHW
80	-	17	-	1	-	-	-	18	-	.972	-	.028	-	0.01
81	6	92	3	5	-	-	1	107	.028	.925	.014	.033	-	0.27
82	4	131	1	17	-	-	-	153	.014	.922	.004	.060	-	0.92
83	1	139	1	14	-	-	-	155	.003	.949	.003	.045	-	0.46
84	-	119	7	9	-	2	1	138	-	.920	.040	.040	-	4.13
85	2	84	7	12	-	1	1	107	.009	.884	.042	.065	-	0.24
86	2	160	3	23	-	-	-	188	.005	.926	.008	.061	-	1.22
87	3	137	5	10	2	-	-	157	.010	.936	.016	.032	.006	0.73
88	-	51	1	6	-	-	1	59	-	.924	.008	.068	-	1.05
Total	18	930	28	97	2	3	4	1082	.008	.926	.016	.049	.001	0.25
H.W	16.0	927.8	32.1	98.2	2.0	0.3	2.6							

J	AB	BB	BC	BD	BX	CC	DD	N	A	B	C	D	X	GHW
80	-	29	1	2	-	-	-	32	-	.953	.016	.031	-	0.08
81	-	10	-	3	-	-	-	13	-	.885	-	.115	-	0.22
82	-	15	-	1	-	-	-	16	-	.969	-	.031	-	0.02
83	-	-	-	-	-	-	-	-	-	-	-	-	-	-
84	-	-	-	-	-	-	-	-	-	-	-	-	-	-
85	-	4	-	2	-	-	-	6	-	.833	-	.167	-	0.24
86	2	35	-	6	-	-	-	43	.023	.907	-	.070	-	0.45
87	-	17	1	3	-	-	-	21	-	.905	.024	.071	-	0.23
88	-	31	1	2	-	-	-	34	-	.906	.047	.047	-	0.07
Total	2	141	3	19	0	0	0	165	.006	.927	.009	.058	.000	1.01
H.W	1.8	141.8	2.8	17.7	-	0.0	0.6							

PEPT

F	AB	AD	BB	BC	BD	BE	BX	CC	CD	CE	CX	DD	DE	EE	XD	N	A	B	C	D	E	X
80					3				1			30	1			35		.043	.014	.929	.014	
81			1		1				2			29				33		.045	.030	.925		
82				1	4				3			36	2			46		.054	.043	.881	.022	
83					3				5			33				41		.037	.061	.902		
84					2				2			11				15		.067	.067	.866		
85		1			5			1	1			69				77	.007	.035	.021	.937		
86		1			6				6			40				53	.009	.057	.057	.877		
87					4				1		1	24				30		.067	.033	.883		.017
88									1			16	1		1	19			.026	.922	.026	.026
Total		2	1	1	28			1	22		1	288	4		1	349	.003	.044	.037	.907	.006	.003
	0.1	1.9	0.7	1.1	27.9	0.2	0.1	0.5	23.4	0.2	0.1	287.1	3.8	0.0	1.9							

PEPT

M	AB	AD	BB	BC	BD	BE	BX	CC	CD	CE	CX	DD	DE	EE	XD	N	A	B	C	D	E	X
80					1				4			26				31		.016	.065	.919		
81					3				2			28	1			34		.044	.029	.912	.015	
82					1							21	3			25		.020		.920	.060	
83				1	1				2			36				40		.025	.038	.937		
84					1							4	1			6		.083		.834	.083	
85		1			9	1			3			90	2			106	.005	.048	.014	.923	.010	
86					3				1			55	1		1	61		.030	.010	.940	.010	.010
87					1							23			2	26		.019		.943		.038
88					5				2			29				36		.069	.028	.903		
Total		1		1	25	1			14			312	8		3	365	.001	.037	.021	.925	.012	.004
	0.0	0.7	0.5	0.6	25.0	0.3	0.1	0.2	14.2	0.2	0.1	312.3	8.1	0.1	2.7							

PEPT

P	AB	AD	BB	BC	BD	BE	BX	CC	CD	CE	CX	DD	DE	EE	XD	N	A	B	C	D	E	X
80												19				19				1.00		
81					11				5	1		102	2			121		.045	.025	.918	.012	
82					10			1	7	1		145	6			170		.029	.029	.924	.018	
83					6				12			145	2			165		.018	.036	.940	.006	
84		3			4				13			122	2			144	.010	.014	.045	.924	.007	
85		2		1	8				7	1		103	3			125	.004	.036	.036	.912	.012	
86		1		1	24				15	1		157	3			202	.002	.062	.042	.887	.007	
87				3	10				10			148	2			173		.038	.038	.918	.006	
88				1	7				2			57			1	68		.059	.022	.912	.007	
Total		6		6	80			1	71	4		998	20		1	1187	.003	.036	.035	.914	.010	.001
	0.1	6.5	1.5	3.0	78.1	0.9	0.1	1.5	75.9	0.8	0.1	991.6	21.7	0.1	2.1							

PEPT

J	AB	AD	BB	BC	BD	BE	BX	CC	CD	CE	CX	DD	DE	EE	XD		A	B	C	D	E	X
80					3				2			26	1	1		33		.045	.030	.880	.045	
81												12	2			14				.929	.071	
82		1			1							13	2			17	.029	.029		.883	.059	
83																						
84																						
85												6				6				1.00		
86	1	1			2				4			36	1		1	46	.021	.021	.043	.893	.011	.011
87					1				3			21				25		.020	.060	.920		
88					5				3			30				38		.066	.039	.895		
Total	1	2			12				12			144	6	1	1	179	.008	.034	.034	.899	.022	.003
	0.1	2.6	0.3	0.3	10.9	0.3	0.1	0.2	10.9	0.3	0.0	144.7	7.1	0.1	1.0							

APPENDIX 2 Chemicals used in the DNA and protein surveys

<u>Reagent</u>	<u>Supplier</u>	<u>Order No.</u>
Acetic Acid	M & B	L723
Agar (Bacteriological No. 1)	Oxoid	-
Agarose LE (Sea Kem)	FMC	50005
Amido Black 10B (Naphthol Blue Black)	Sigma	N3005
L-Amino Acid Oxidase	Sigma	A5147
3-Amino-9-ethyl carbazole	Sigma	A5754
Ampicillin	NBL	070608
Bacto Tryptone	Difco	0123-01
Bacto Yeast Extract	Difco	1896-17
Biogel P-60	Biorad	150/1603
Boric Acid	Fisons	B3800
Blue Dextran	Sigma	D5751
Bovine Serum Albumin	Sigma	A4503
Bromocresol Purple	BDH	20014
Bromophenol Blue	BDH	20015
Citric Acid	Sigma	C7129
Chloramphenicol	Sigma	C0378
Chloroform	BDH	10077
α - ³² P CTP	Amersham	PB10162
α - ³² P dCTP	Amersham	PB10205
Deoxyribonucleic Acid (calf thymus)	Sigma	D1501
DNA (λ)	BRL	520 5250 SA
DL-Dithiothreitol (DTT)	Sigma	D0632
Eco RI	NBL	010106
Ecoscint (Emulsifier-Safe)	Packard	-
Ethanol	Various	-

Ethidium Bromide	Sigma	E8751
Ethylene Diamine Tetra Acetic Acid (EDTA) disodium salt	Fisons	D0700
Ficoll 400	Pharmacia	17/0400/01
D-Glucose	BDH	10117
Hae III	NBL	011407
Heparin 5000 I.U/ml	Weddel Pharm.	-
Hind III	NBL	011806
Hoechst dye 33258	Polyscience	9460
HCl	M & B	L730
Iso-amyl alcohol	Fisons	A6960
DL-Isocitric Acid (Na ₃ Salt)	Sigma	I1252
L-Leucyl-glycyl-glycine	Sigma	L9750
L-Leucyl-L-tyrosine	Sigma	L0501
Li OH	BDH	29073
Lysozyme	Sigma	L6876
MgCl ₂	BDH	29096
MTT	Sigma	M2128
Nick Translation Kit	BRL	8160 SB
Nicotinamide-adenine dinucleotide phosphate (NADP)	BDH	42051
Nigrosin	Sigma	N4754
Peroxidase (horse radish)	Sigma	P8250
Phenazine methosulphate (PMS)	Sigma	P9625
Phenol (crystalline)	BDH	10188
6-Phosphogluconate (Na ₃ Salt)	Sigma	P7877
Polyvinylpyrrolidone	Sigma	P5288
Potassium acetate	Fisons	P3760
Proteinase K	Sigma	P0390
RNAase inhibitor	Promega	P211

T7-RNA polymerase	Boehringer	881767
Skimmed Milk Powder	Sainsburys	-
Sodium acetate	BDH	10235
Sodium azide	BDH	10369
Sodium chloride	M & B	L944
Sodium citrate	M & B	L908
Sodium Dodecyl Sulphate	BDH	44215
Sodium Dodecyl Sulphate	Pierce	28364
NaH ₂ PO ₄	M & B	L949
Na ₂ HPO ₄	Fisons	S4501
NaOH	BDH	30167
Spermidine	Sigma	S2626
Starch	Sigma	S4501
Transcription Kit	Promega	P1121
Tris hydroxymethylamino methane (TRIS)	Sigma	T1378
Triton X-100	Sigma	T6878
X-ray developer	Kodak	LX24
X-ray fixer (Hypam)	Ilford	438608
Xylene cyanole FF	Sigma	X2751

APPENDIX 3 Solutions required for DNA fingerprinting

DNA Extraction (solutions made up in sterile distilled water = SDW)

<u>20 x SET</u>	3M	NaCl
	1M	Tris
	20mM	EDTA Na ₂
		pH to 8.0 with HCl
<u>Proteinase K</u>	10mgml ⁻¹	Proteinase K
<u>SDS</u>	25%	w/v
<u>TE</u>	10mM	Tris
	1mM	EDTA Na ₂
		pH to 8.0 with HCl

DNA Electrophoresis (solutions in distilled water)

<u>5 x T.B.E.</u>	0.445M	Tris
	0.445M	Boric Acid
	0.002M	EDTA (pH 8.0)
<u>50 x T.A.E.</u>	2M	Tris
	2M	Glacial Acetic Acid
	0.05M	EDTA (pH 8.0)

Bromophenol blue loading dye (BPB)

20%	Ficoll
0.2M	EDTA Na ₂
0.25%	Bromophenol blue
0.25%	Xylene cyanol FF

Southern Blotting (solutions in distilled water)

<u>Depurination solution</u>	0.2M	HCl
<u>Denaturing solution</u>	1.5M	NaCl
	0.5M	NaOH

Alkaline Transfer solution A.T.S.

1.5M NaCl

0.25M NaOH

Neutralising solution 3M NaCl

0.5M Tris

pH to 7.0 with HCl

20 x SSC 3M NaCl

0.3M Sodium citrate

Hybridization

Nick-stop mix 0.9% Blue dextran

0.03% Bromocresol purple

20mM EDTA

solution in T.E.

10 x BLOTTO 10% skimmed milk powder

0.2% sodium azide

in SDW

5 x Denhardts 0.5gm Polyvinylpyrrolidone

0.5gm BSA

0.5gm Ficoll

in 500ml SDW

Fluorometric Assaying

10 x TNE 100mM Tris

10mM EDTA

1M NaCl

pH 7.4 with HCl

Demographic study of a wild house sparrow population by DNA fingerprinting

Jon H. Wetton, Royston E. Carter, David T. Parkin & David Walters

Department of Genetics, Medical School, Queen's Medical Centre, Nottingham NG7 2UH, UK

Over the past twenty years, several techniques from biochemical and molecular genetics, such as enzyme electrophoresis and isoelectric focusing, have been widely and successfully applied to the study of population differentiation and evolution¹. However, they have been less applicable to demographic problems such as assigning parentage to individuals within a population. This stems from a general weakness of data derived from enzyme loci: allele frequencies at polymorphic loci are sufficiently skewed that the majority of individuals are of one or two genotypes. Many enzyme systems can only be examined post mortem, so that the loci are of little use if the animals are to be studied in the wild. The search for new and more sensitive techniques for detecting genetic variation has continued, and recently a major discovery has come from molecular biology. Jeffreys *et al.*² have reported the detection of a type of hypervariable 'minisatellite' DNA that is extraordinarily polymorphic in human populations. We have applied their technique to several bird species and particularly to a population of house sparrows (*Passer domesticus*) near Nottingham. We report here that one of the human minisatellite clones is a suitable probe for sparrow DNA and that it reveals variation as extensive as that found in man. These results suggest that analysis of minisatellite DNA will be a powerful tool in the study of demographic population genetics.

The study population of house sparrows lives around farm buildings at Brackenhurst College of Agriculture³. It has been under observation since 1979 and currently there are 80 nest boxes, of which about 60 are occupied each year. During the breeding season, the boxes are monitored at two-day intervals, and all nestlings that reach 10 days of age are ringed and blood samples are taken. Adult birds are trapped in the colony with nets, and also at the nest during breeding. Every adult bird is given a unique combination of colour rings, and a blood sample is taken if the bird has not been handled before. The use of colour rings, combined with direct observation, allows the identification of mated pairs, and of their attendance at nests during the incubation and feeding of young, without the need for continuous disturbance.

A survey of the population was undertaken using starch gel electrophoresis of seven polymorphic loci. This shows that approximately 8% of nestlings are genetically mismatched with at least one of their putative parents (ref. 3 and J.H.W., unpublished data). The allele frequencies are such that we would expect to detect about half the cases in which non-parental nest attendance occurs. However, in common with similar studies, the data are poor and errors are large. To illustrate the power of polymorphic minisatellite analysis in resolving familial

relationships, we will describe the results from two separate nest boxes in the house sparrow population during 1985 and 1986.

The first family consists of a male A who was paired with female B during 1985, and then with three different females (C, D and E) successively through 1986. Young birds were fledged successfully from each breeding attempt, giving rise to families of three (B1-B3), two (C1-C2), four (D1-D4) and three (E1-E3) offspring. The enzyme data give no evidence of any mismatch between parents and offspring that could be assigned to infidelity or egg-dumping (data not shown). DNA was extracted from whole blood and prepared as described in Fig. 1. After treatment with *Hae*III, electrophoresis, Southern blotting and hybridization with the 33.6 minisatellite probe from Dr Alec Jeffreys, the autoradiograph shown in Fig. 1 was produced. We can distinguish a total of about 60 bands with some confidence, of which the four unrelated females have an average of 14.25 (s.e.m. = 0.85) each. Comparing these four individuals in pairs shows that the probability that a fragment present in one is also present in another is 0.086. The probability that all fragments present in one individual are present in another is thus: $0.086^{14.25}$ ($<10^{-14}$). As with humans, house sparrows are extremely variable in their minisatellite phenotypes.

Closer inspection of the bands in Fig. 1 shows that the pattern of inheritance is the same as that reported by Jeffreys *et al.*^{2,4}. Thus, every band present in one of the offspring occurs in at least one parent. Analysis of a larger family suggests that there is little or no linkage: this is not particularly surprising because Bulatova *et al.*⁵ have shown that there are at least 38 pairs of

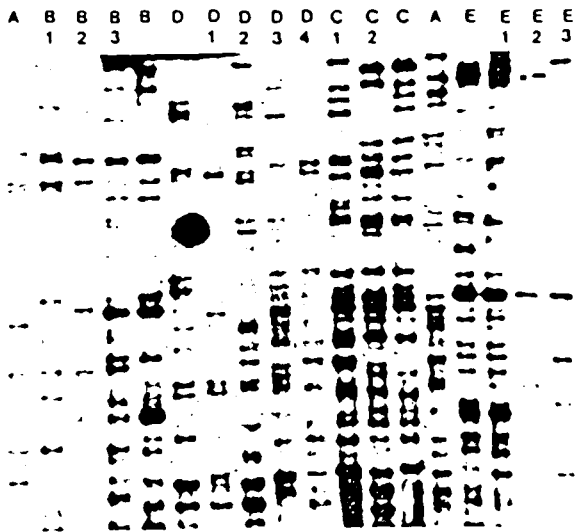


Fig. 1 DNA 'fingerprints' of four broods of house sparrows from a single nestbox that have a common father (A) but different mothers (B, C, D and E).

Methods. Whole blood (25 µl) collected by jugular venipuncture was suspended in 475 µl SET buffer (0.15 M NaCl, 0.05 M Tris, 1 mM EDTA pH 8.0) and stored at -80 °C. Subsequently, 15 µl proteinase K (10 mg ml⁻¹) and 7.5 µl 25% w/v SDS were added to the thawed sample and it was incubated overnight at 55 °C. DNA was purified by three extractions with phenol, followed by two with phenol/chloroform and one with chloroform, and was precipitated with 0.1 vols 3M sodium acetate and 2 vols absolute ethanol at 0 °C, pelleted at 11,600g for 5 min, washed with 75% ethanol and vacuum dried. The DNA was dissolved in 150 µl TE (10 mM Tris, 1 mM EDTA). DNA (~8 µg in 8 µl) was cut with 10 U *Hae*III overnight at 37 °C, then electrophoresed through a 22 cm 0.7% agarose gel for 65 h until all fragments <3 kilobases had migrated off the gel. DNA was then dephosphorylated by treatment with 0.2 M HCl, denatured *in situ* and transferred by Southern blotting to Schleicher and Schuell BA 85 nitrocellulose membranes. High specific activity (>8 × 10⁸ c.p.m. µg⁻¹) 33.6 probe was prepared by nick translation of 33.6 RF DNA with [³²P]dCTP and [³²P]dGTP (3,000 Ci mmol⁻¹). Hybridization was carried out overnight at 65 °C in 1.5 × SSC and 5 × Denhardt's soln. The blot was washed at 65 °C in 1.5 × SSC, 0.1% SDS. Filters were autoradiographed for four days at -80 °C using two intensifying screens.

Table 1 Similarity coefficients *D* calculated from the results in Fig. 1

		Sets of offspring			
		B1-B3	C1-C2	D1-D4	E1-E3
Adults	A	0.577 (0.105)	0.453 (0.021)	0.535 (0.033)	0.615 (0.028)
	B	0.628 (0.017)	0.144 (0.085)	0.175 (0.045)	0.169 (0.036)
	C	0.077 (0.039)	0.687 (0.040)	0.156 (0.059)	0.092 (0.061)
	D	0.040 (0.020)	0.247 (0.065)	0.480 (0.064)	0.172 (0.059)
	E	0.201 (0.052)	0.066 (0.001)	0.160 (0.056)	0.506 (0.118)

Values shown are means, with s.e.m. in parentheses.

conclusion of parentage. Every band present in G occurs in one or other (or both) of its presumed parents. Assuming that the bands are inherited independently, the probability of this occurring by chance among unrelated birds is extremely low.

It is apparent from these results that the polymorphic minisatellite DNA discovered by Jeffreys *et al.* in humans and also reported in cats and dogs⁷ is equally variable in other species. It appears to be inherited in the same simple fashion and individuals are as different from one another as are people. Its use provides an excellent means for resolving relatedness in nature, and is likely to revolutionize the study of those aspects of behaviour, population genetics and biometry that require a detailed demographic knowledge of populations.

We are grateful to Dr A. J. Jeffreys for the minisatellite probe and for his helpful attitude to this research. We thank him and

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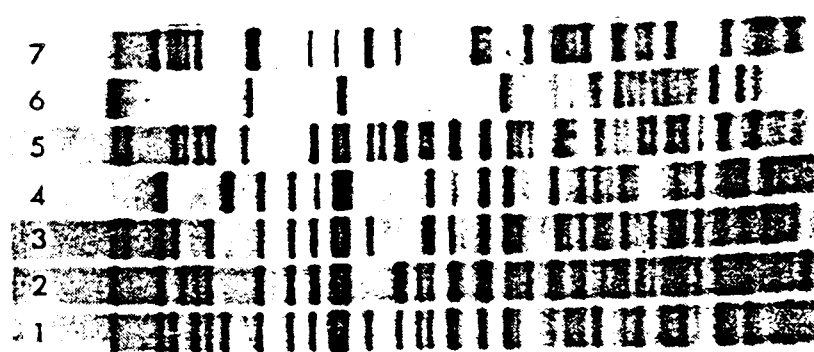
Improved genetic fingerprinting using RNA probes

R.E.Carter, J.H.Wetton and D.T.Parkin

Department of Genetics, University of Nottingham, Queens Medical Centre, Nottingham NG7 2UH, UK
Submitted April 4, 1989

The minisatellite containing polylinkers from 33.6 and 33.15⁽¹⁾ were liberated from RF form DNA⁽²⁾ by sequential digestion with Hind III and EcoRI. The fragments were purified by electroelution prior to T₄ DNA ligation into pSPT 18 and 19⁽³⁾ vectors, from which the polylinker had previously been removed by Hind III and EcoRI.

RNA probes are prepared from purified DNA (miniprep) according to manufacturers instructions (BCL, Promega and Amersham) by transcription from the T7 promoter with the following modifications: α -³²P CTP (400 Ci.mmol⁻¹) is used, incubation is at 38.5°C for 60', and substrate DNA is not digested with DNase I. Unincorporated radionucleotides are removed by P60 spun column chromatography.



Genetic fingerprints of seven House Sparrows (Passer domesticus) revealed by RNA probe pSPT 19.6. Three micrograms of Hae III restricted DNA from three sibs (1-3), their parents (4 & 5) and two unrelated adults (6 & 7) were

electrophoresed through 22 cm. 0.8% agarose gel until fragments smaller than 3 Kb were lost. DNA was transferred to a nylon membrane by alkaline Southern blotting, prehybridised and hybridised in 1 x SSC, 1% SDS, 1 x Blotto (1% Marvel milk powder, 0.01% Na Azide)⁽⁴⁾ at 65°C and washed in 1 x SSC, 0.1% SDS at the same temperature. Autoradiography was for 4 hours with two intensifying screens then 4 days without screens.

The Jeffreys' probes 33.6 and 33.15 and pSPT derivatives are the subject of patent No. GBA 2166445 and worldwide patents (pending) for commercial diagnostic use.

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