CHROMOSOMAL VARIATION OF THE COMMON SHREW *SOREX ARANEUS* L. IN BRITAIN

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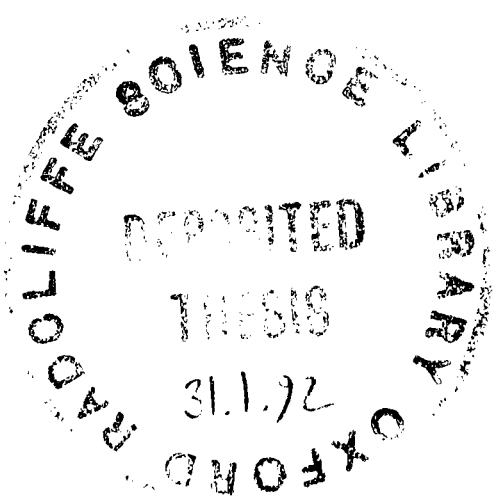

ABSTRACT

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Chromosomal variation of the common shrew *Sorex araneus* L. in Britain

Throughout the range of the common shrew (*Sorex araneus* Linnaeus 1767), repeated Robertsonian fusion mutations have led to a karyotypic polymorphism dividing the species into chromosome races. Studies of fertility were undertaken in the male, both of homozygotes and of heterozygotes forming meiotic multivalents of varying complexity.

Observations made at pachytene, diakinesis/metaphase I and metaphase II did not provide evidence for fertility impairment in homozygotes or simple heterozygotes. Males forming a chain of seven chromosomes during meiotic prophase I were produced through a program of captive breeding, and were brought to premature sexual maturity through photoperiod manipulation. In these more complex heterozygotes, incomplete pachytene pairing was frequently observed (68% of cells), germ cell death was found to be elevated (23%), and data from analysis of metaphase II spreads are indicative of an increase in nondisjunction above background of approximately 10%.

At the interface between two chromosome races, a complex of clines of chromosome frequency can be found. Field studies determined the routes and widths of four such clines (*ko*, 8.6km; *kq*, 28.4km; *no*, 37.1km; *pr*, 47.9km), and concluded that the courses taken are entirely consistent with maintenance through a mechanism of heterozygote disadvantage. Estimates of disadvantage made from cline width were in close agreement with those derived from the fertility studies.

DECLARATION

As the supervisor of Mr Mercer, I certify that the majority of this thesis is his own work. Those parts attributable to other workers are listed in the preface and the authors acknowledged in the text.

Dr JB Searle

PREFACE

The faculty board of Biological Sciences allow the submission of a thesis as a series of related papers, and I have chosen this form in order to publish the results of my work as rapidly as possible. As a consequence of this, some of the chapters have been written in a style for submission to conference proceedings (chapters two, three and five), whilst others are written in a style for publication in a journal (chapters four and six and appendix 1). The text of each chapter is unchanged from the manuscript with the exception of references to other parts of the thesis, and the format of individual chapters varies in accordance with the requirements for submission to the journals and conference proceedings concerned.

I would also like to state that the following data in this thesis are not my own work, and have been included here either either in support of my own data, or to increase the size of the dataset and therefore more clearly demonstrate the conclusions;

Chapter 2
- part of table 1 (see footnote)
- table 2

Chapter 3
- table 1
- table 2
- some data in table 3 (see footnote)
- figure 1

Chapter 4
- Scoring of Haematoxylin and Eosin-stained tubules; Figure 3

Chapter 5
- data from the Muthill site (table 1)

Chapter 6
- table 1b

Appendix 1

I bred the animals for this study, but did not take any part in the experimental work, or the writing of this paper.

I would like to acknowledge the following;

My supervisor, Dr JB Searle, whose close supervision and meticulous attention to detail have been invaluable. Drs C Beechey, S Garagna and BMN Wallace for teaching me experimental techniques; Mr M Amphlett for animal trapping and photography. Miss C Everett, Miss P Stockley and Mr S araneus for their assistance in animal breeding, trapping and feeding. During my field trips, laboratory accommodation was generously provided by Dr J Flowerdew and Mr A Hilton (Cambridge), Professor G Hewitt (Norwich) and Dr D Bryant and Mr S Mc Leod (Stirling). This work was supported by the Natural Environment Research Council, grant number GT4/87/TLS/9.

CONTENTS

CHAPTER 1

INTRODUCTION

NOMENCLATURE AND CONVENTIONS

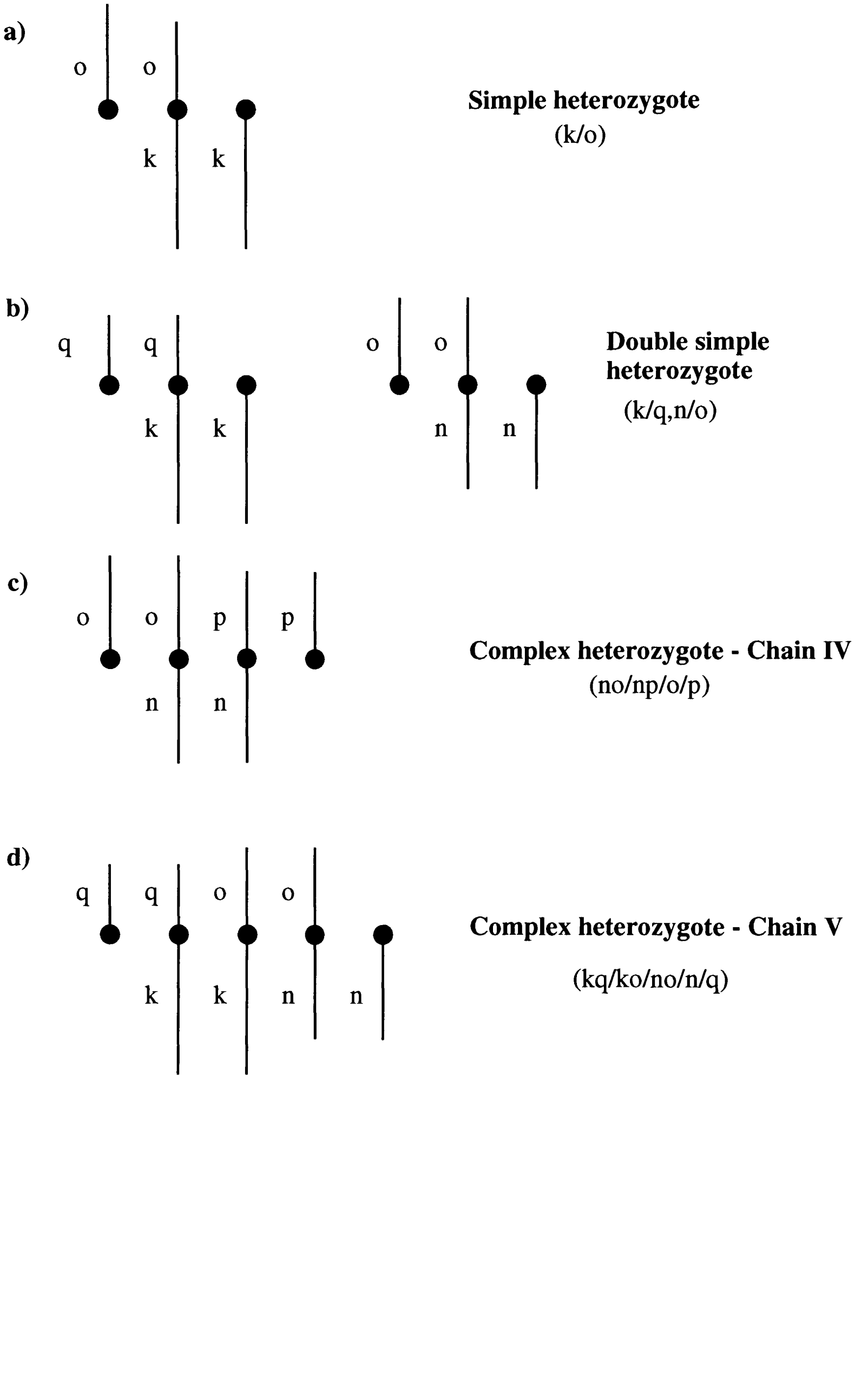
Chromosomes may be categorised on the basis of the position of the centromere. Those with an interstitial centromere (and therefore two chromosome 'arms') are here called metacentrics, whilst those with a terminal or near-terminal centromere (and therefore one major 'arm') are termed acrocentric. The common shrew *Sorex araneus* Linnaeus 1767 exhibits a widespread chromosomal polymorphism due to a repeated joining of acrocentric chromosomes to form metacentrics by a mechanism known as Robertsonian fusion. The resultant complex patterns of chromosomal variation require a standardised nomenclature, and the conventions adopted here are the proposals of the International *Sorex araneus* Cytogenetics Committee (ISACC), as put forward in the Proceedings of the Second International Conference on the Cytogenetics of the Common Shrew (Searle et al. 1991), held in Arzier, Switzerland, in September 1990. The following recommendations are of especial note. The Y sex chromosomes are designated Y_1 and Y_2, with the former being the smaller element, agreeing with established practice but contrary to the recommendations of the Denver Conference (1960). Chromosome arms are referred to throughout as italicised lower-case letters of the alphabet, ranked in approximate order of size, with *a* representing the largest. Different elements are separated by commas, thus for the individual, *af* refers to the largest metacentric element found in the karyotype, whereas *k*,*o* refers to the two acrocentric chromosomes homologous to the metacentric *ko*. In a case of Robertsonian heterozygosity, Searle et al. suggest the use of a solidus (/) to separate the chromosomes involved (for

example, an individual possessing the metacentric *ko* and the two acrocentric homologues *k* and *o* could be described by *ko/k/o*, or more simply *k/o* -- figure 1a).

The widespread occurrence of populations in which a distinctive assemblage of metacentrics may be found has led to the subdivision of the species into 'chromosome races', and the ISACC have made some recommendations relating to these. The precise definition of a chromosome race is not stipulated, but a race is here defined as a group of populations monomorphic for the same metacentric chromosomes (there may be regional variants of a race in which other metacentrics are fixed or polymorphic). It is becoming increasingly common to follow the practice of naming a race after the type locality, although other conventions are still in use. The ISACC require a common evolutionary origin for all populations of the same race, and that different populations of the same race do not possess a monobrachial homology (see below). The ISACC provide a standard race-defining karyotypic nomenclature, which is very similar to that for the individual.

Different races are found in different parts of the range of the common shrew, and where two races meet (or two variants of the same race), populations polymorphic for a metacentric, or possessing metacentrics with the same arms but in different combinations, may be found. In the simplest case where only one Robertsonian metacentric is involved, any animal possessing one metacentric and its two acrocentric homologues is called a 'simple' Robertsonian heterozygote (figure 1a). In cases where polymorphism affects two or more chromosome pairs, the result is a multiple simple Robertsonian heterozygote

Figure 1. Different types of Robertsonian heterozygote



(e.g, *k/q,n/o* -- figure 1b). In a case where populations possessing different metacentric chromosomes homologous for only one arm meet, individuals possessing both of these metacentrics are said to have a monobrachial homology (for example if an animal possessed both *no* and *np*, expressed as *no/np/o/p* in the standard nomenclature -- figure 1c), and termed a complex heterozygote. Considering that monobrachial homology cannot exist within a chromosomal race (see definition above) this animal must also qualify as an inter-racial hybrid. Further complexity is possible if more than two chromosomes share an arm in common, and an example of this would be an animal with *ko,kq,no* (*ko/kq/no/n/q*), also a complex heterozygote (figure 1d). A final concept to introduce is that of the hybrid race. In some cases, the most plausible origin of a race is through the introgression into one population of subsets of two different sets of race-specific metacentrics such that the resulting population has monobrachial homology with both of the donor races. Examples include the Abisko and Hattsjö races of Northern Sweden (Fredga 1987).

ROBERTSONIAN MUTATION

A variety of sequences of breakage and reunion could lead to the formation of one metacentric chromosome from two acrocentric precursors, and many proposals for a mechanism of Robertsonian fusion have been put forward without a molecular base. These involve breakage in the pericentric region, the precise positions of the breakpoints giving rise to a differing loss of chromosome material (Meylan 1970; John and

Freeman 1975), but a change in DNA content is usually not detected (Redi et al. 1990).

Breakage and reunion can give rise to dicentric fusion products if the breakpoint on both acrocentrics lies on the short arm. This may lead to inactivation of one centromere (Rattner and Lin 1985; Maraschio et al. 1991), or if the two centromeres are sufficiently close together, the forces acting on each will be sufficiently similar to promote stability, even if both remain active (Hsu et al. 1975).

Among those theories with a molecular base, that of Stahl et al. (1983) most closely reflects classical cytogenetic theory, with pericentric breakage and reunion involving nonhomologous acrocentrics whilst aggregated during prophase. It would also involve some DNA loss, which as already stated, is rare. A second theory (Holmquist and Dancis 1979), does not require DNA loss or breakage, and will therefore produce a dicentric. It relies instead on mutation of a terminal hairpin sequence of the intermediate formed by telomeric recombination (see Blackburn and Szostak 1984 for review) prior to replication. This model of telomere replication has the advantage of addressing the Okasaki dilemma in which the 5' end of a DNA strand cannot be replicated by any known polymerase, and also providing an elegant mechanism for telomere-telomere fusion. In common with the model of Stahl et al. and 'classical' theory, metacentric formation by this method should involve arms throughout the chromosome complement at random, but although this seems to be the case in *Mus* (Bauchau et al. 1988), there is evidence for non-random involvement of arms in Robertsonian fusion in humans (Hecht

and Kimberling 1971). The model of Redi et al. (1990), proposes that fusion may occur between acrocentrics with regions of satellite DNA showing a high degree of homology. These regions may occasionally undergo meiotic pairing with nonhomologous chromosomes, and if this is followed by cutting and ligation of the resultant heteroduplex, a dicentric with very little DNA loss could form. Since no genes are lost in this model, the impact of this type of mutation on selection would be minimal.

Dover et al. (1984) speculate that there may be a genetic element involved in the formation of Robertsonian metacentrics in some species, through a process of occasional non-Mendelian segregation of genetic material termed 'molecular drive'. This speculation is based on the observed tendency in some mammalian populations for repeated Robertsonian fusions. Dover et al. propose that if a gene or genes predisposed their 'host' chromosome to undergo Robertsonian fusion, then subsequent transference of such a sequence to another chromosome would result in multiple Robertsonian fusions in populations possessing such sequences. Repeated formation of the same metacentric in a population could also aid its fixation.

If we accept the models of Robertsonian fusion not involving chromosomal breakage, then this implies a high proportion of dicentric metacentrics in populations possessing Robertsonian mutations. Whether or not one of the centromeres was inactive, the presence of two simplifies a mechanism of subsequent Robertsonian fission, and helps to explain the existence of acrocentric-bearing populations surrounded by

populations in which metacentrics have reached fixation. This may be particularly pertinent in the common shrew (see chapter 6). Otherwise, there is little clue as to the specific mechanism of Robertsonian fusion in this species, except that whatever the mechanism is, it occurs repeatedly.

EVOLUTIONARY HISTORY OF THE *SOREX ARANEUS* GROUP

The common shrew, *Sorex araneus* Linnaeus (1758), is a small and highly active insectivore with a continuous distribution extending throughout northern and central Europe (including Britain) eastwards to the river Yenisei and lake Baikal in Asia (Corbet 1978; Hausser et al. 1985). In western Europe, isolated populations are found in the Massif Central and the Pyrenees in France, and the Appennines in Italy.

Work by Sharman (1956) revealed the sex chromosome system to be XX (female) and XY_1Y_2 (male), due to a fusion of the ancestral X with an autosome. At diakinesis during meiosis, the autosomal arm of the metacentric X chromosome forms one to three chiasmata with the autosomal Y_2, whilst the 'true' Y_1 is terminally associated with the ancestral arm of the X (Fredga 1970; Searle 1986b).

The XX/XY_1Y_2 sex-determination system serves as evidence of a common ancestry for the group of species known as the *Sorex araneus* complex. First proposed on the basis of morphology by Hoffmann (1971), modified on cytological grounds (Meylan and Hausser 1973), and further strengthened on the basis of biochemical data (Catzeflis et al. 1982; Hausser et al. 1985; George 1988), the monophyletic origin of this group is no longer in doubt. The validity of some of the species

have been the subject of debate, but the *Sorex araneus* group is now generally accepted to comprise *Sorex araneus, S. coronatus, S. granarius, S. arcticus, S. tundrensis* sensu lato, *S. daphaenodon, S. caucasicus,* and *S. asper.* Detailed karyotypic study has been confined to the first five of these. The small genetic differences between species (Catzeflis et al. 1982; George 1988) argue strongly for a recent divergence, and (in concert with palaeontological data-- Reumer 1989) a Eurasian origin within the last 500,000 years (Hoffmann and Peterson 1967). The same authors propose the first theory for the diversification of this group, in which a primitive soricid stock colonised Eurasia and the Beringian 'land-bridge' between the Palearctic and nearctic during the Riss Pleistocene glaciation. The subsequent interglacial caused the flooding of Beringia and the division of the ancestral type into a palearctic and a nearctic stock. The former evolved into *S. araneus, coronatus* and *granarius,* whilst the latter was the ancestral stock of *S. arcticus.* Beringia re-emerged in the subsequent (Würm) glaciation, and colonisation by an *arcticus*-derived stock resulted in the evolution of the two subspecies *S. tundrensis,* which then spread to colonise both the palearctic and nearctic.

A dependence on climate and habitat preference for diversification in this model is supported by Reumer (1989) who proposes climate as the major factor in the periodic rises and falls in species diversity of the Soricidae. The more recent model proposed by Volobouev (1989) argues that the European members of the *S. araneus* group (*araneus, coronatus,* and *granarius*) are not derived directly from a primitive stock

and that *tundrensis* is the possessor of the most primitive karyotype of the *Sorex araneus* group. Again, Volobouev proposes the Beringian refugium as an important element in speciation, but with a *granarius*-like ancestral stock becoming divided between the palearctic and the nearctic during the Riss, with the North American form differentiating into *Sorex arcticus arcticus* and *S.a. maritimensis*, and with the Eurasian form giving rise to *S. araneus, granarius* and *coronatus*. *Sorex tundrensis* independently diverged from the ancestral form by two centromere shifts and four centromere-telomere translocations, spreading to the nearctic during the Würm.

In Europe, Schaefer (1975) found that *S. araneus* and *S. daphaenodon* were morphologically distinct and present in the Tatra mountains of Czechoslovakia 30,000 years BP (Before Present), and so at least some of the current diversity predates the most recent glaciation. The *granarius*-like ancestor underwent one Robertsonian fusion to form the metacentric *tu*. During the Riss, populations of these animals became isolated in the Iberian peninsula, and one of these, *Sorex granarius*, has maintained this highly acrocentric karyotype (with the exception of a polymorphism for a pericentric inversion in *tu*-- Wójcik and Searle 1988). Another population underwent a further Robertsonian fusion to form the metacentric *af*, and this stock in turn underwent diversification, due to subdivision into European glacial refugia during the Würm in a similar manner to that in Beringia (Searle 1984). This led to two centric shifts and five Robertsonian fusions forming *Sorex coronatus*, which subsequently spread to colonise the European lowlands from the

Pyrenees to the Rhine, and *Sorex araneus*, which through the acquisition of a large number of Robertsonian metacentrics has become one of the most karyotypically diverse species known.

AUTOSOMAL POLYMORPHISM IN *S. ARANEUS*

Autosomal Robertsonian polymorphism was first identified in the common shrew by Sharman (1956), in individuals caught near Oxford in 1955. Ford et al. (1957) subsequently identified the elements involved as numbers six, seven, and eight in their nomenclature, and a further element, number four, was added by Ford and Graham (1964). In Switzerland and France, the work of Meylan (1964; Meylan and Hausser 1973) indicated that there were a number of different karyotypes for the common shrew, and although these were at first designated as races A and B, race A was later given specific status as *Sorex coronatus* Millet, 1828. No hybridization has been recorded between this species and *S. araneus*, despite the existence of a zone of sympatry in Western Switzerland (Neet 1987), and a small degree of introgression of *coronatus*-specific alleles into local *S. araneus* populations (Neet and Hausser, 1991).

In studies in the late 1960s and early 1970s, new races were identified by conventional staining (Fredga 1973; Orlov and Koslovsky 1969). Not until chromosome banding methods were introduced to identify chromosome arms (Halkka et al. 1974) was the extensive polytypy in this species revealed (Reumer and Meylan 1986).

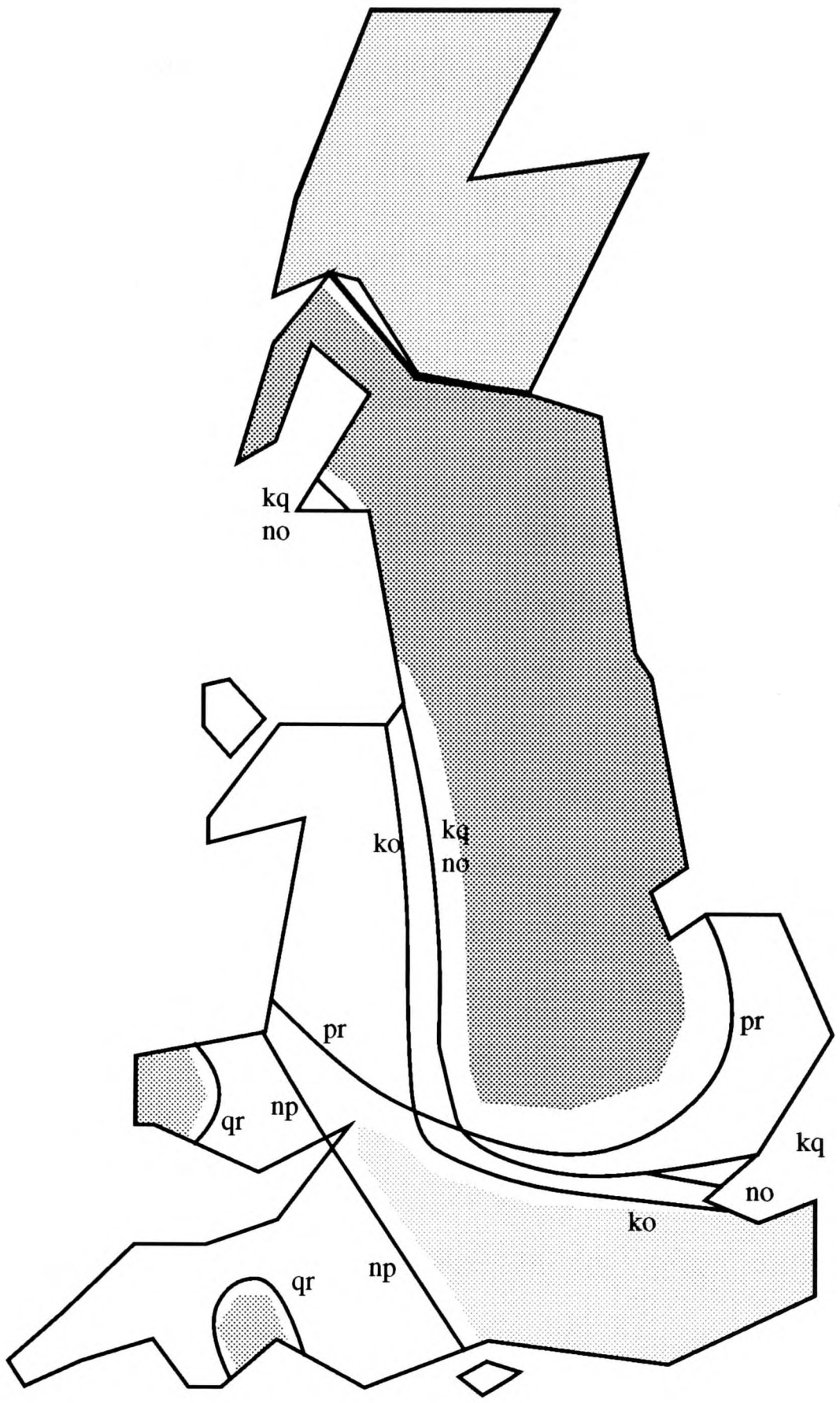
The current known chromosomal variation in the common shrew reflects in microcosm the diversity seen in the *Sorex*

araneus complex. The majority of chromosome races are in Europe west of the USSR (probably a reflection of the intensity of research and not their actual distribution). A phylogeny for these races was proposed by Searle (1984), and divides the races of *S. araneus* into three phylogenetic groups, the Siberian, West European and East European, which presumably reflect the subdivision of the species into refugia during the last glacial maximum. As with chromosome races, these groups are characterised on the basis of the possession of common Robertsonian metacentrics. The fusion *hi* is found in all the races of the West European Phylogenetic group, with *hn* in the East European, and the fusions *mn* and *gk* characterise those of the Siberian group.

KARYOTYPIC DIVERSITY IN BRITAIN

With the exception of the earliest work, the study of karyotypic variation of the common shrew in Britain has been undertaken by Searle and collaborators (see Searle 1988 for review), and in terms of the distribution of chromosome races, Britain is one of the best known areas of the range. As first described (Searle and Wilkinson 1987), three chromosome races were defined: the Aberdeen race, occupying Northern Scotland, Cornwall and Southwest Wales, the Hermitage race, with a distribution running in a belt diagonally Northwest to Southeast from Cheshire to Kent, and the Oxford race, extending throughout the Midlands, East Anglia and Northern England (see figure 2). A re-evaluation of this scheme (Searle et al. 1990) casts doubt as to whether the Hermitage race should be regarded as a distinct race, but instead as an

Figure 2. Distribution of chromosome races of the common shrew in Britain



Key:

Monomorphic races:

Aberdeen race

Oxford race

Hermitage race

The Oxford-Aberdeen hybrid zone

pr 50% isocline of metacentric frequency

intermediate between the Aberdeen and Oxford races within the complex southern hybrid zone. Taking the view that there are only two valid chromosomal races in Britain, their karyotypes can be given as;

'Oxford' race: XX/XY_1Y_2 *af,bc,hi,gm,jl,kq,no,pr/p,r,tu*

'Aberdeen' race: XX/XY_1Y_2 *af,bc,hi,gm,jl,ko,np,qr/q,r,tu*

With the Hermitage race considered as a hybrid form. In addition to this, a low-level Robertsonian polymorphism affecting arm combination *jl* is found throughout Britain.

ROBERTSONIAN HETEROZYGOSITY AND MEIOTIC IRREGULARITY

Examples in the mouse, Man and in many other species have shown that heterozygosity for a Robertsonian rearrangement may have an effect on fertility, although this shows considerable variation between individuals, and due to age, sex, species, genetic background, and the chromosomes involved (examples include Martinez-Castro et al. 1984 in Man; Mittwoch et al. 1990 in female *Mus* and Mahadevaiah et al. 1990 in the male; Searle, in press for other mammals). Possible causes of impairment may be either prezygotic or postzygotic (death of chromosomally unbalanced offspring).

Prezygotic impairment

Karyotypic heterozygosity, including Robertsonian heterozygosity, may be associated with the death of germ cells prior to meiosis (Garagna et al. 1990 for the female; Chandley

1988 for the male), reinforcing the view that structural change may be paralleled by alterations in gene expression. But the major causes of prezygotic impairment are regarded as those mechanisms operating at meiosis, and directly attributable to a structural abnormality.

The causes of increased germ cell death in karyotypic heterozygotes are widely debated. Two main hypotheses have been proposed: those of Forejt (1979) and Miklos (1974). The 'X-inactivation' hypothesis of Forejt is itself a modification of the model of Lifschytz and Lindsley (1972) and revolves around the fact that the sex chromosomes in the male are inactive during meiosis and that the heteromorphic configurations in karyotypic heterozygotes show regions which are not properly paired during pachytene. In this model, nonhomologous prophase pairing between the unpaired region of the X chromosome and the heteromorphic autosomal configuration interferes with X inactivation. This results in inappropriate gene expression, and the subsequent death of the affected germ cell. Evidence to support this is strong in some cases (e.g. *Mus*, Grao et al. 1989), but equivocal in others (e.g, sheep, Chapman and Bruére 1977; *Mus*, Mahadevaiah et al. 1990). Inappropriate expression of X-linked genes has been detected electrophoretically in *Mus* by Hotta and Chandley (1982), and it would therefore seem that this mechanism may account for germ cell death in some cases.

There are a number of important qualifications to this mechanism, the most obvious is that it can only be applied to the male.

The mechanism proposed by Miklos (1974) and modified by Burgoyne and Baker (1984) is applicable to both sexes, and states that a number of critical pairing sites exist throughout the genome. Unless these become saturated by homologous pairing during prophase I of meiosis, germ cell death will result due to inappropriate gene expression. This mechanism has the obvious advantage that it is equally applicable to both sexes, and is often invoked in cases where germ cell death has no other obvious cause. However it is a mystery as to why gene expression should be affected by the paired or unpaired state of the chromosomes.

Postzygotic impairment

In Robertsonian heterozygotes, postzygotic impairment stems from the production of aneuploid gametes by anaphase I nondisjunction. In mammals, no autosomal monosomies, and virtually no autosomal trisomies survive beyond birth (the exceptions being trisomies 13, 18 and 21 in man, and some extremely rare instances in other mammals -- references in Searle 1989). The production of hyper- or hypoploid gametes, whilst not associated with germ cell death (Ford and Evans 1973, but see also Redi et al. 1983), will result in a reduction of litter size.

As with germ cell death, the rate of nondisjunction seems to vary widely, with no concensus emerging as to the mechanism. The spindle is expected to be best adapted to the symmetrical disjunction of bivalents, and therefore errors are more likely to occur when multivalents are present (Daniel 1988). Nijhoff (1981) suggests that long or rigid multivalent

structures (such as found in Robertsonian heterozygotes) in the equatorial plate may encourage nondisjunction. Univalence, whether due to asynapsis or desynapsis at meiotic metaphase I is expected to predispose to nondisjunction (de Boer 1986 -- although it may also lead to germ cell death), and in man, the greater degree of univalence of chromosome 21 has been attributed to desynapsis caused by a relatively small number of chiasmata (Henderson and Edwards 1968). Asynapsis has also been suggested as a more likely cause of increased chromosome 21 nondisjunction, through analysis of recombination frequencies (Warren et al. 1987). Crossovers very close to the centromere between the metacentric and acrocentric elements in a multivalent have also been identified as a possible source of nondisjunction (Cattanach et al. 1976). Hansson (1979) suggests association of satellite regions of homologues may promote segregation failure.

The delay in meiosis common to structural heterozygotes may enhance nondisjunction, at least in the male (Hansmann et al. 1988), whilst in the female it has been suggested that nondisjunction may be promoted by nucleolar persistence during the long dictyotene stage (Vagner-Capodano et al. 1987). Females produce more aneuploid gametes than males (Plachot et al. 1987; Martin et al. 1991), and in view of the shortened reproductive life of the heterozygote female, a contributory factor to this may be an increasing frequency of segregation errors towards menopause (Chandley 1985).

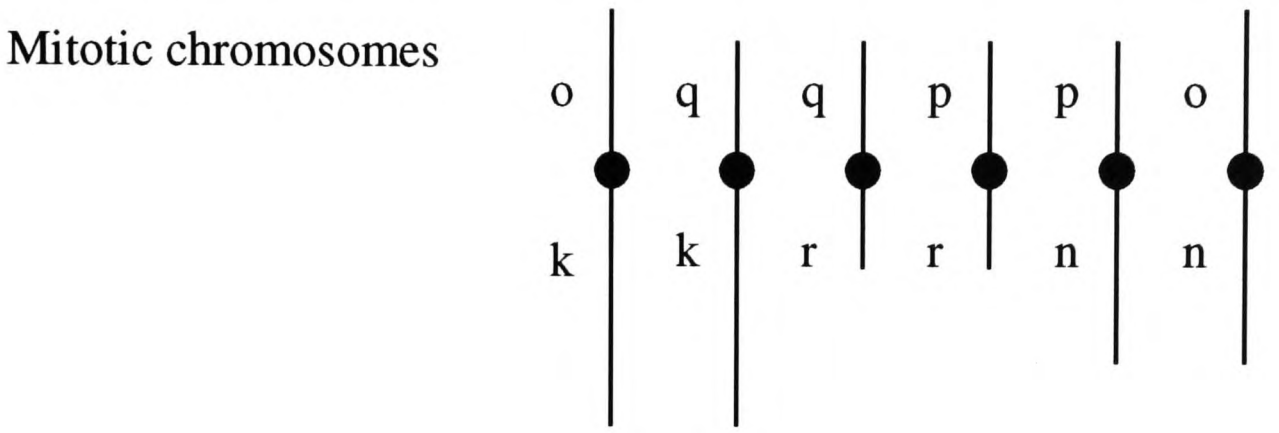
Interchromosomal effect

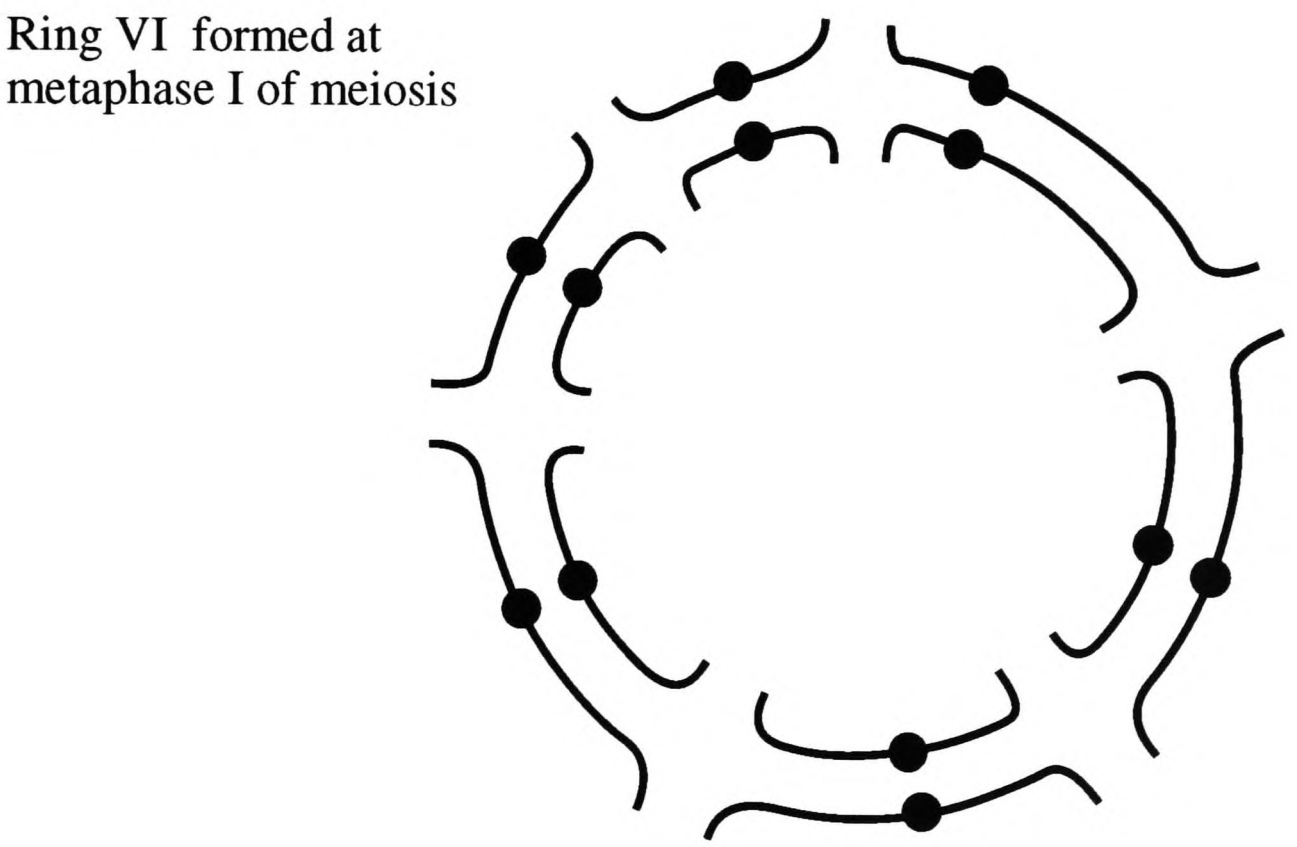
There is evidence that mutations affecting one element of the karyotype may cause meiotic irregularities in other elements, at least in Man (Davis et al. 1985; Farag et al. 1987). In the case of the Chinese hamster, however, this claim has been convincingly refuted (Sonta and Kitayama 1991), and therefore such an effect may be species dependent.

The effect of rings and chains

As already explained, meiotic pairing in inter-racial hybrids of the common shrew will result in the formation of multivalents which may take the form of a chain (figure 1d), or in some rare instances, a ring (for example hybrids between the Aberdeen and Oxford races -- figure 3). A similar range of configurations have been generated in the laboratory mouse (Gropp and Winking 1981; Gropp et al. 1982; Winking 1986), and they display a considerable range of nondisjunction frequencies and spermatogenic impairment due to germ cell death. It seems from these studies that ring configurations have a greater effect on nondisjunction, whilst chains more profoundly affect germ cell death. The explanation proposed in the case of chains is that asynapsis of the proximal regions of the acrocentric elements may result in interference with the sex bivalent and germ cell death in the mode of Forejt, as discussed above. It would appear that chain length is an important determinant of the extent of germ cell death, and so in addition we must invoke some mechanism dependant for its severity on the length of the chain formed. In the case of ring configurations, nuclear reconstructions by Wolf and

Figure 3. A complex Robertsonian heterozygote forming a ring VI at metaphase I of meiosis





Winking (in de Boer and de Jong 1989) have shown profoundly abnormal cytoarchitecture with an alteration in the number and disposition of heterochromatic blocks around the nucleus. These abnormalities leave open the possibility of an increase in nondisjunction due to any of the mechanisms described above.

CLINES AND HYBRID ZONES

The form of the boundary between two karyotypically different populations of the same species will depend upon the differences in karyotype and the effects of these differences on the heterozygotes formed at the interface. Three possible types of interface are considered.

A simple cline

A cline of frequency for a single metacentric may be found between two racial variants, such as between populations of the Oxford race of the common shrew in East Anglia (see chapter 6). In this case, a cline of frequency the metacentric *pr* is found.

The width of a cline for a particular Robertsonian metacentric chromosome is primarily determined as a balance between the effects of selection and of dispersal on heterozygotes (Barton 1979). Heterozygous animals may be assumed to suffer fertility impairment relative to either homozygote (see preceding section), and populations with a high degree of heterozygosity may therefore be expected to produce proportionately less offspring than surrounding populations. This gives rise to a 'hybrid sink', a constant

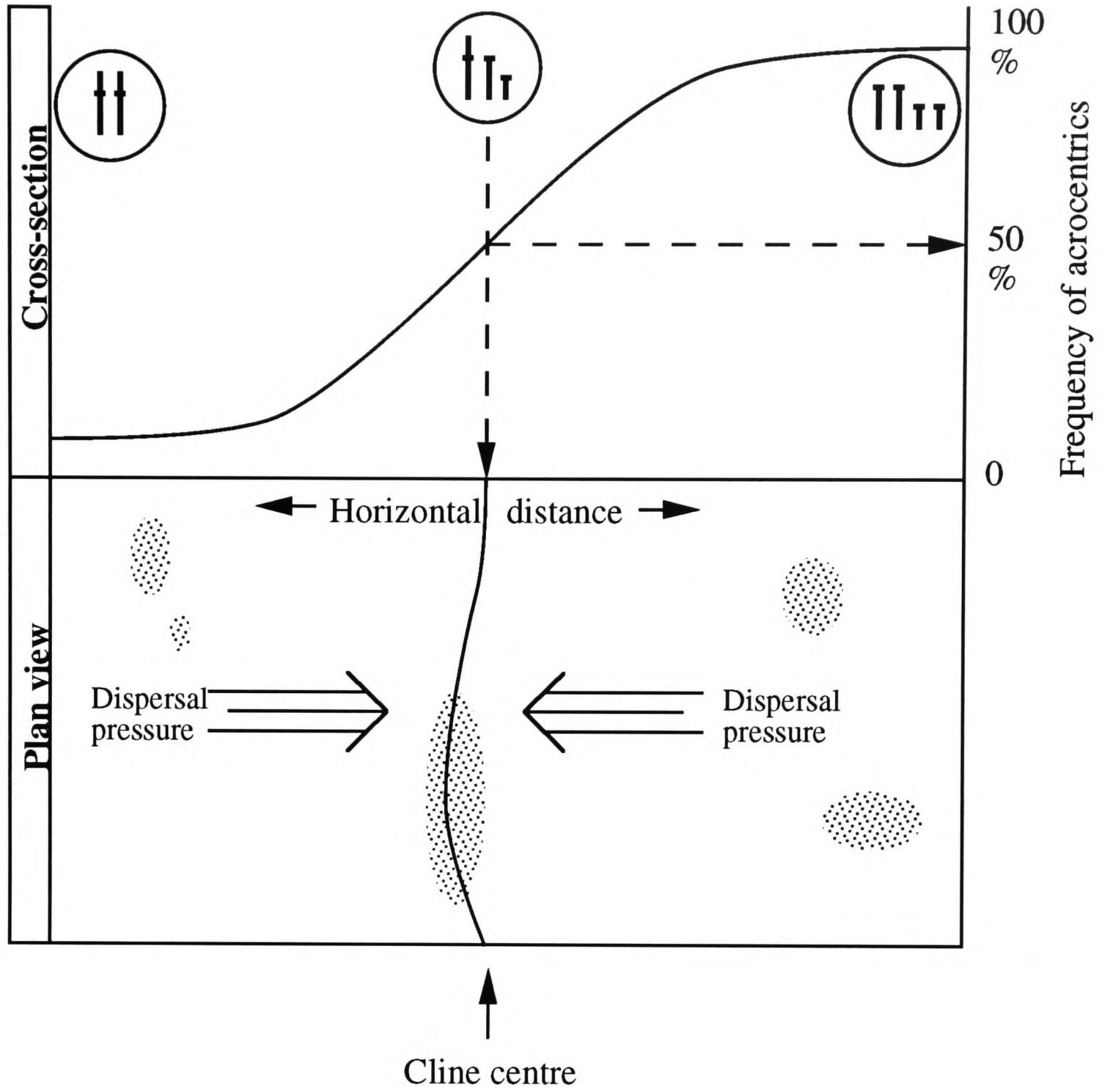
net inflow of animals from the monomorphic flanking populations towards the centre of the cline (figure 4). The extent to which this influx is balanced by emigration from the affected populations and a consequent tendency to widen the zone will be dependant upon the degree to which fertility is impaired in heterozygotes; the milder the fertility decrease, the wider the cline.

Although the internal mechanisms affecting fertility operate independently of external factors, the dispersal distance of the common shrew and the densities of populations of different karyotype will be related to the environment. Where the cline runs through homogeneous habitat, these forces will act to equalise the length presented to either bordering population, and it will therefore tend to run in straight lines. This form is known as a 'tension zone' (Key 1968). The situation changes where different habitat types support different population densities. If racial variant 'A' happens to occupy habitat which can support a higher density of shrews than the adjacent variant 'B', the cline at the interface between these two variants will receive a higher influx of 'A'-type animals than those of type 'B', causing movement of the cline into the territory of 'B'. The cline will stabilise wherever the influx of animals from both sides into the centre equalises.

Multiple clines

In cases where racial variants differ by several metacentrics, the interface between them will consist of more than one cline of the form described above. Each of these will

Figure 4. A simple cline



Key

Poor habitat

Predominance of metacentric homozygotes

Predominance of heterozygotes

Predominance of acrocentric homozygotes

have a width determined by the degree to which heterozygosity of that particular Robertsonian fusion affects fertility. In these circumstances, a range of factors will affect the relationships between clines. Any cline with another adjacent to it will have the influx of animals on one side reduced through proximity to the hybrid sink of its neighbour, and this influence will act to draw clines together. In such a case, if the reduction in fertility due to the presence of an additional heterozygous arm combination is significant, then cline widths will be reduced.

Populations within an aggregation of two or more clines will be capable of producing homozygotes as well as a range of single and multiple simple heterozygotes. Making the assumption that heterozygosity for several metacentrics incurs a relatively more severe penalty than expected from heterozygosity for one, selection will favour homozygosity in these populations, producing a tendency for clines to separate (Barton and Bengtsson 1986). The effects of habitat have also to be taken into account, and if clines are similar in width, then it is likely that they will associate in a similar manner with features in the habitat through which they run, and this may enhance the influences causing the aggregation of clines. If clines of radically different width pass through the same habitat, then it is possible that they will become associated with different geographical features, and therefore enhance the effects of separation.

Monobrachial homology

The interface between two populations possessing a monobrachial homology by definition separates different races of the common shrew, and may properly be called a hybrid zone. In the simplest case, where the only difference between races is a single monobrachial homology, then the clines of chromosome frequency will of necessity be joined at the 50% isocline (figure 5a). Populations at the centre of such a zone will produce hybrids with monobrachial homology and a correspondingly severe fertility depression, leading to a very narrow zone and steep clines. In terms of movement, the zone will behave in the same manner as a simple cline. The presence of such a powerful hybrid sink would attract clines relating to other, non-monobrachial differences between the races, to give the complex appearance of the typical common shrew hybrid zone. A number of zones of this form in the common shrew are further elaborated by the presence of a central 'acrocentric peak' (figure 5b), a high frequency of acrocentric elements localised to the centre of the zone, and not found in either bordering race (Searle 1986a; Fredga 1987; Fedyk 1986). The presence of these acrocentrics will reduce the frequency of inter-racial hybrids possessing one or more monobrachial homology, and will therefore act to increase the fitness of the population as a whole.

AIMS AND PRACTICAL DIFFICULTIES

The complexity of the chromosomal polymorphism in the common shrew raises questions concerning the origins of this system and its implications for the evolution of the species.

Figure 5.

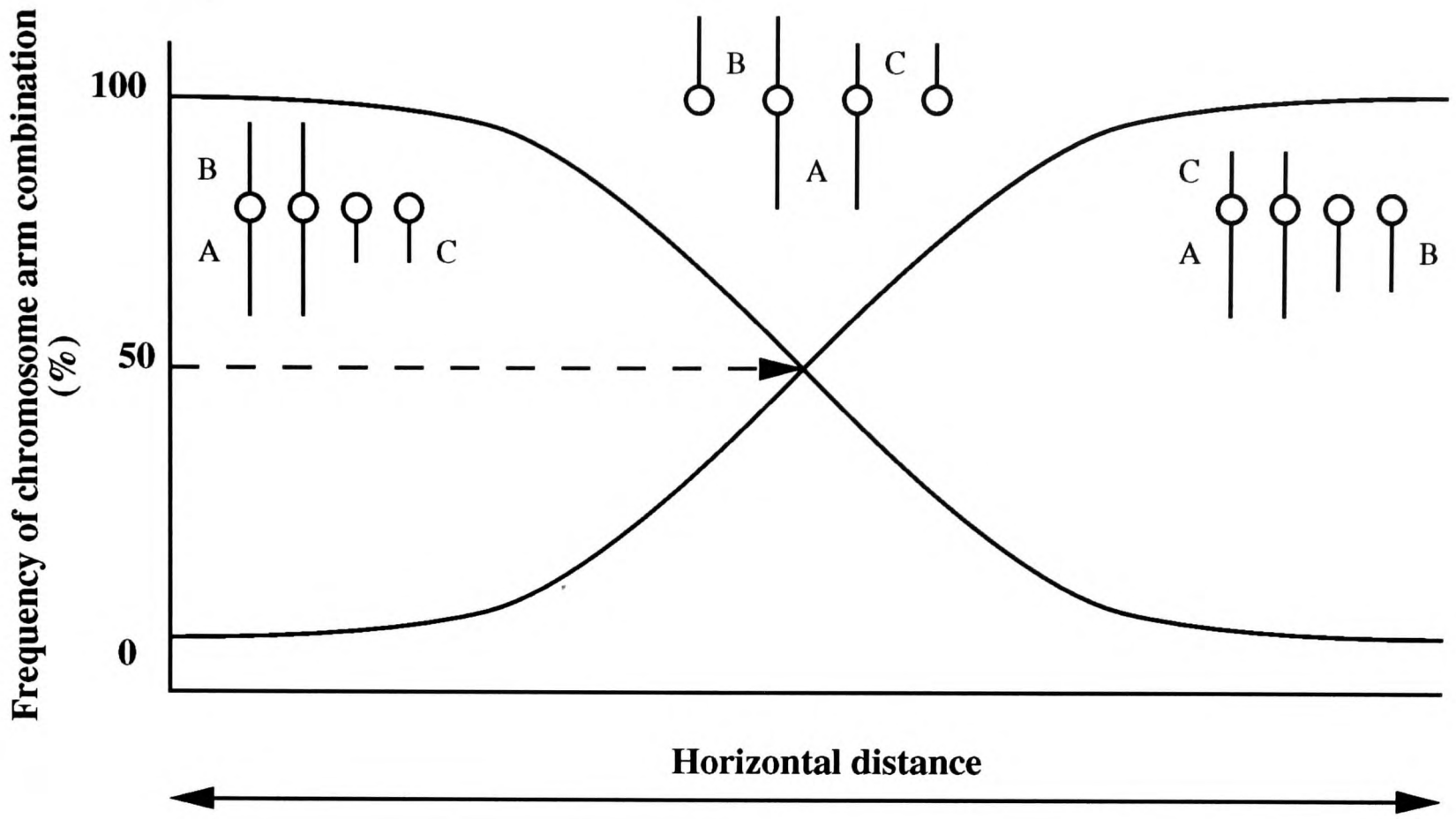
Two intersecting clines of frequency for the hypothetical metacentric chromosomes *AB* and *AC*, which share an arm in common (a monobrachial homology).

a) If no acrocentric elements are present, then the two intersecting clines are necessarily 'locked' together at the 50% isoclines, and a high frequency of the relatively unfit heterozygotes with monobrachial homology (*AB/AC*) will be formed in the central populations.

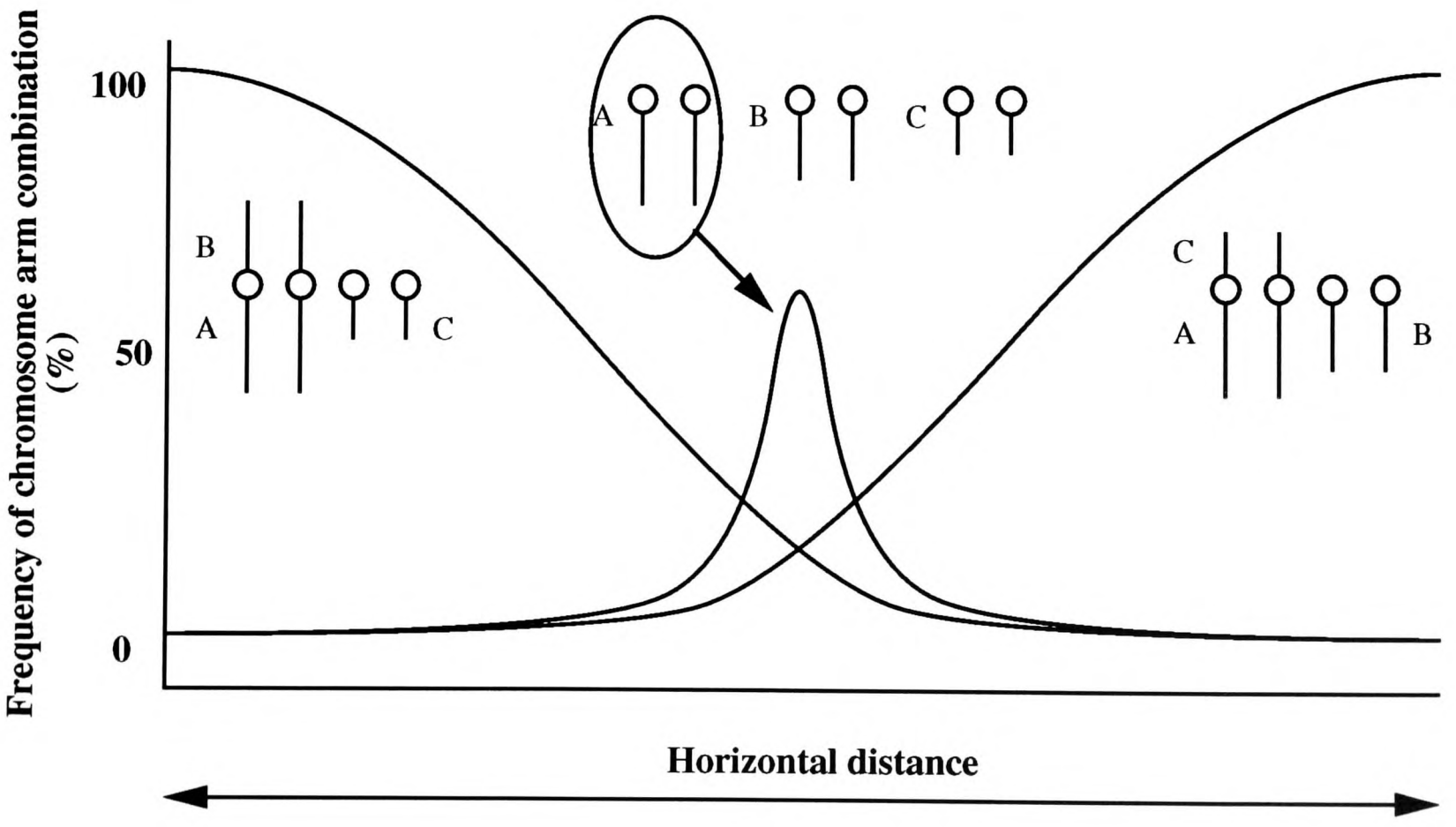
b) If the acrocentric homologue of the chromosome arm common to both metacentrics (*A*) is present, it will reduce the frequency of heterozygotes with monobrachial homology, and therefore increase the overall fitness of the central populations. This acrocentric will consequently be maintained at a high frequency in this region and form an acrocentric peak.

Figure 5. Two clines with monobrachial homology

a)



b)



It is the aim of this thesis to examine the effects of this Robertsonian polymorphism in the individual, and to attempt to define and quantify the basis of the heterozygote disadvantage. This investigation involved the examination of common shrews of different races and karyotypes at key stages of gamete production, confined to the male in order to acquire a sufficiently large sample. In order to examine the most complex hybrid karyotypes which are extremely rare in the wild, it has been be necessary to generate them through a programme of captive breeding coupled with artificial sexual maturation.

In parallel with the laboratory studies on gamete production, a number of field studies have been undertaken of interfaces between populations of different karyotype. Three areas have been selected for study, the case of a simple cline is represented by the cline for the chromosome *pr* in East Anglia, the well known Oxford-Hermitage hybrid zone (Searle 1986a) from the Chiltern hills in the East to the Severn valley in the west provides an example of a hybrid zone in which complex monobrachial hybrids may be expected and an acrocentric peak is known, and the hybrid zone expected between the Oxford and the Aberdeen races, in the region of Stirling in central Scotland was expected to produce hybrids which should form a ring multivalent at meiotic prophase I.

Sampling was performed both to construct cross-sections of all clines to calculate gradient and width, and also to map the paths of individual clines to determine the extent of their agreement with cline and hybrid zone theory.

Determination of the degree of fertility depression incurred by different types of heterozygote through cytological analysis will allow predictions to be made of the width of clines and hybrid zones. These can then be compared with the cline widths determined in the field, to test the hypotheses regarding the maintenance of chromosomal clines, and chromosomal polymorphism as a whole, in the common shrew in Britain.

There are two main difficulties associated with this project, the first is the relative unsuitability of the common shrew as a laboratory animal. With regard to breeding and maintaining the common shrew in captivity, the reputation for fragility of this animal appears to be well deserved, reducing the sample sizes of many experiments to a size insufficient for statistical analysis. Although the mitotic index of the common shrew is high and routine karyotyping has been easy, all attempts have failed at producing reliable cytological methods for the study of germ cells, and in all cases a success rate of 25% was the best achieved, leading to small sample size and the necessity to combine some of the datasets with published data for analysis. The second problem is more general to small mammals in comparison to insects and plants. Insufficient data can be collected to provide resolution of minor features within the cline, and hence the cline widths and paths determined have a margin of error.

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CHAPTER 2

CAPTIVE BREEDING OF THE COMMON SHREW (*SOREX ARANEUS*) FOR CHROMOSOMAL ANALYSIS

S.J. Mercer and J.B. Searle.

ABSTRACT.-- A method is described for systematic breeding of wild-caught common shrews (Sorex araneus). In studies over four breeding seasons, a total of 294 animals from 54 litters were known to have been born, 243 of which were successfully weaned. These animals and their parents provided data concerning litter weights and sex ratio of offspring, as well as chromosomal and genetic segregation. By manipulation of photoperiod, sexual maturation of laboratory-born males was induced. It should now be possible to maintain a continuously-breeding laboratory colony for genetic analysis and other studies.

BACKGROUND

The common shrew, Sorex araneus, has always been regarded as difficult to maintain and breed in captivity (see Searle 1984a). One of the first attempts at captive maintenance was that of Adams (1912), but successful breeding was not achieved until the late 1940's by Dehnel (1952). Although common shrews have been bred in captivity intermittently since then (Crowcroft, 1957, Vogel, 1972, Vlasak, 1973, Fedyk, 1980), nobody has established a long-term self-sustaining colony of the type routinely maintained for shrews of the subfamily Crocidurinae (Hellwing, 1971; Oda et al., 1985). In this paper, we describe our methods for the breeding and maturation of common shrews, which we believe would allow such a colony to be established.

Our breeding study extended over four seasons, during which time data were collected on litter size, sex ratio and weight of young, condition of the mothers and response of laboratory-reared young to photoperiod. In addition, shrews of different chromosomal constitutions were crossed to generate inter-racial hybrids and to study the inheritance of different chromosomal morphs in this highly variable species.

MATERIALS AND METHODS

Common shrews were bred during 1980, 1981 (J.B.S.), 1988 and 1989 (S.J.M.). The procedures adopted by J.B.S. have already been described (Searle, 1984a); thus we concentrate herein on the more recent methods of S.J.M.

Animal collection.-- Wild shrews were collected with unbaited Longworth traps (Chitty and Kempson, 1949) at sites characterised by known frequencies of chromosomal types (Searle 1983). Females were collected pregnant (late April, May), or prior to their first pregnancy (early April). Mature males were collected at the same times as the females.

Animal maintenance.-- After capture, shrews were kept for up to a week in standard household buckets containing 1 to 2cm of moist, milled peat or garden soil, with a covering of hay for concealment and as nesting material. Approximately 25g of moist, minced ox heart was provided twice daily. Individuals were subsequently transferred to individual, open-air enclosures. These enclosures varied from 0.13 to 0.7m^2 in basal area (only the larger of these were used for mating or

rearing of young) with sides at least 45 cm high. They had a substrate of peat or garden soil, and turf sods were provided as additional cover. Any attempt to keep the turf moist (see Dehnel, 1952 and Searle, 1984a) resulted in an infestation of mites, which declined when watering was discontinued. At least one nest box was provided, consisting of an inverted six-inch plantpot filled with hay. In the majority of enclosures, a wire or plastic exercise wheel was provided, and all enclosures had wire mesh tops to prevent loss of animals to predators.

For long-term maintenance and for breeding, 25g of a complex meat-based diet (Searle, 1984a) was provided twice daily. More food was given to lactating females and to other animals as their individual needs demanded. Water was provided ad lib.

Breeding.-- Pregnant and lactating females were left undisturbed as much as possible, with a notable increase in food consumption taken to indicate the approximate onset of lactation. Thus for wild-conceived pregnancies, twenty-three days after such increased food consumption (the length of lactation-- Dehnel, 1952; Searle, 1984a), the enclosure was first examined for the presence of young, which were considered weaned if their teeth had erupted. Additional signs of weaning were increased activity in the enclosure and disturbance of the food bowl, frequently coupled with vocalisation.

At weaning, all animals were removed from the enclosure, weighed, sexed (Searle, 1985a), and any distinctive features

noted. Female offspring were generally killed and karyotyped soon after weaning to give a guide to the karyotypes of the parents and their male siblings. Siblings lived peaceably together in the same cage if the number of nest boxes provided was at least equal to the number of individuals. After removal of her young, the adult female was immediately exposed to a mature male in her original enclosure. The male was left with the female for 20 days (the gestation period-- Dehnel, 1952, Searle, 1984a), and then removed to prevent infanticide (in contrast to the method of Genoud and Vogel, 1990, where males were re-introduced before birth to mate at the postpartum estrus). For these captive crosses, the enclosure was examined for a weaned litter 23 days after removal of the male.

Collection of data.-- Animals were killed by cervical dislocation, weighed, examined for signs of white spotting of the fur, and sex was confirmed by dissection. Body and tail lengths were measured using a variant of Morrison-Scott's pin method (Jewell and Fullagar, 1966). Mitotic preparations were made from bone marrow using the air-drying method of Ford (1966), modified for the shrew by Searle (1983). Mitotic preparations were G-banded by a combined ASG and trypsin method (Searle, 1983), and at least five spreads were scored using the light microscope at 1000X under oil immersion.

Photoperiod studies.--Nineteen immature males born and reared in captivity were used to assess the effect of photoperiod manipulation on the induction of physical and sexual maturity. At the beginning of this experiment, the age

(from weaning) of the animals was between 52 and 69 days, and the ambient photoperiod was approximately 14.5L:9.5D.

For long daylengths (LD), individuals were kept in an animal room in cages, either singly or in small groups, and maintained at a light regime of 16L:8D. For short days (SD), the light regime was 8L:16D, and photoperiod cabinets were used (Grocock and Clarke, 1974). In these cabinets, shrews were housed singly in polypropylene rat cages (350mm by 200mm by 200mm deep), lined with a layer of peat or soil, and the bars replaced with a sheet of perforated metal. Each cage was provided with an exercise wheel, a nest box and a dish of water. Food was provided twice daily as described above.

The males were subjected to one of three photoperiod regimes. Animals subjected to the first regime (regime A, $\underline{n}$=5, 2 months LD) were killed before the others to provide an early indication of response to long photoperiods. All of the individuals exposed to regime A derived from one litter, in contrast to those exposed to regimes B and C, which derived from five and six litters respectively. Animals under regimes B and C were kept for four months, one set of animals (regime B, $\underline{n}$=6) subjected to LD only, whereas the other (regime C, $\underline{n}$=8) had two months SD followed by two months LD. At the end of their photoperiod treatment all animals were killed, and in addition to the normal measurements, fresh weights of testes and seminal vesicles were also recorded.

RESULTS AND DISCUSSION

Success of the breeding method.-- Altogether, 294 young from 54 litters were known to have been born (table 1). Of these, 243 (44 litters, $\overline{X}$=5.5) survived to weaning, including 150 young (24 litters, $\overline{X}$=6.3) from natural conceptions and 93 young (20 litters, $\overline{X}$=4.7) from crosses in captivity; the maximum litter size recorded at weaning was 10. The difference in size between wild and captive-conceived litters was not significant. No difference in litter size was detected between inter- and intra- racial crosses (see also Searle 1984b).

There were strong indications that it is better to bring females into captivity as pregnant animals and cross them after the first litter rather than to attempt to breed nulliparous females. Of 10 wild-caught pregnant females collected between the 24th April and 22nd May 1988, all succeeded in rearing litters, and five subsequently reared litters conceived in captivity. Of five wild-caught nulliparous females collected between 5th and 10th April 1989, none raised a litter to weaning. Of 50 attempted crosses overall, at least 25 resulted in successful fertilization (50%); when nulliparous females are excluded (nine failed crosses), the success rate rises to 61%. Twenty-one of 81 shrews (12 of 49 females) used for breeding died prematurely, the others were killed after weaning their respective litters.

There was a high frequency of preweaning mortality among wild-conceived young in 1980 and 1981 (43% excluding those deliberately killed: table 1), with much of this due to the death of nursing mothers. For wild conceived young in 1988 and 1989 and laboratory-conceived young, there was far less

preweaning mortality. Following weaning, mortality was highest in the first month, at 16% (8 of 49 males). Death was often associated with a disturbance such as movement to another enclosure.

Hair loss was noted in some animals in captivity, usually taking the form of a generalized reduction in the thickness of the fur, especially around the lower back and base of the tail. This was generally associated with dampness in the enclosure, and was frequently improved by replacement of bedding and relocation of the nest boxes. Loss of hair did not seem to affect either longevity or fertility.

Table 1.-- Success of captive breeding in the common shrew

Where conceived	Failed crosses	Pre-weaning: Number of animals (litters) Born[a]	Killed	Died[a]	Weaned	At weaning: Mean litter size	Proportion males
Wild[b]	---	80(15)	8(1)	29(6)	43(8)	5.4	55%
Captive[b]	6[d]	69(14)	7(1)	4(1)	58(12)	4.8	
Wild[c]	---	110(17)	0	3(3)	[f]107(16)	6.7	53%
Captive[c]	10[e]	35(8)	0	0	35(8)	4.4	66%
WILD	---	190(32)	8(1)	32(9)	150(24)	6.3	--
CAPTIVE	16	104(22)	7(1)	4(1)	93(20)	4.7	--
TOTAL		294(54)			243(44)	5.5	57%

[a] Minimum estimates.
[b] Data of Searle (1984b).
[c] Data of S.J.M.
[d] Total of 23 attempted crosses. Three females were killed during gestation.
[e] Excludes crosses with nulliparous females (nine, all failed).
[f] Includes only one complete litter (of a single animal).

* The dataset of SJM is given in full in Appendix B, tables 1 and 2.

Measuring maturity in the male.-- The degree of maturation in these males was assessed in relation to the following data from wild-caught shrews (Stockley and Searle, in press). For immature males, body weights are typically in the range 5-8g (Crowcroft, 1956) and neither the paired testes nor the seminal vesicles weigh more than 5mg (Brambell, 1935b; note that Brambell erroneously names the seminal vesicles 'prostate glands'). Adult males weigh 8-12g, and typically have a combined testis weight greater than 100mg and often exceeding 200mg; likewise for the seminal vesicles. Seminal vesicle weight is a good indicator of androgen activity (Grocock and Clarke, 1974), and their growth therefore indicates the onset of sexual maturation. Body length is given by Crowcroft (1956) as a reliable indicator of physical maturity in the common shrew. Because the tail does not grow perceptibly after weaning (table 2), the ratio between body and tail may be used as an indicator of maturity which takes into account natural variation in overall size. From our data (table 2), body (measured from nose to anus) to tail (measured from anus to tail tip) ratios tend to lie between 1.6 to 1.9 for immatures, and 2.0-2.1 for adults, close to the values that may be extrapolated from Crowcroft (1957).

Table 2.-- Body (including head) lengths and tail lengths for a representative sample of adult and immature common shrews (JB Searle, unpublished data)

Age and sex	n	Lengths (mean ± S.E., in mm) Body	Tail	Body to tail ratio
Adult females	10	82.67 ± 0.69	39.92 ± 0.89	2.1
Adult males	10	83.03 ± 0.50	41.61 ± 0.71	2.0
Immature females				
Litter 1	1	66.5	42.5	1.6
Litter 2	4	75.25 ± 0.71	42.52 ± 0.24	1.8
Litter 3	2	71.65 ± 0.05	40.20 ± 0.20	1.8
Litter 4	2	71.85 ± 1.45	40.05 ± 2.75	1.8
Litter 5	4	69.05 ± 0.46	39.65 ± 0.76	1.7
Immature males				
Litter 1	7	70.96 ± 0.37	43.04 ± 0.49	1.6
Litter 2	2	72.85 ± 1.45	44.80 ± 0.60	1.6
Litter 3	3	72.93 ± 0.57	41.53 ± 0.93	1.8
Litter 4	3	69.93 ± 1.09	37.37 ± 1.10	1.9
Litter 5	4	69.68 ± 0.86	40.38 ± 1.28	1.7

The adults were collected from the vicinity of Oxford (U.K.) during 10th-25th May 1981. The immatures (the progeny of a variety of crosses involving common shrews from the vicinity of Oxford and Aberdeen, U.K.) were reared in captivity and measured within 5 days of weaning from 27th July - 11th August 1981.

Animal weights.-- The mean weight of captive-bred offspring at weaning was 7.99g* (n=115). There was no significant difference, however, between the weights of offspring conceived in the wild and in captivity, or between sexes.

The body weights of nulliparous females were not available for this analysis (see Success of the breeding method, above), but adult female body weights were measured at the weaning of each litter. There was a significant regression between litter size and body weight of the female at weaning for the first litter (n=11, r^2=0.684, P=0.0017, d.f.=10), and the relationship between the size of the second or third litter and body weight of the female at weaning of her previous litter only just fails to achieve significance (n=5, r^2=0.900, P=0.0516, d.f.=4 -- figure 1). The extent to which the first relationship is explained by greater mammary development in females with large litters is unknown. No correlation is evident between the size of the first and second litters (n=6, r^2=0.412, P>0.05, d.f.=5), and the second relationship is therefore taken to suggest that heavier females produce larger litters. No relationship was found between litter size and mean weanling weight, indicating that smaller litter size is not compensated for by larger weanlings.

* The dataset of SJM for offspring weights is given in Appendix B, table 3.

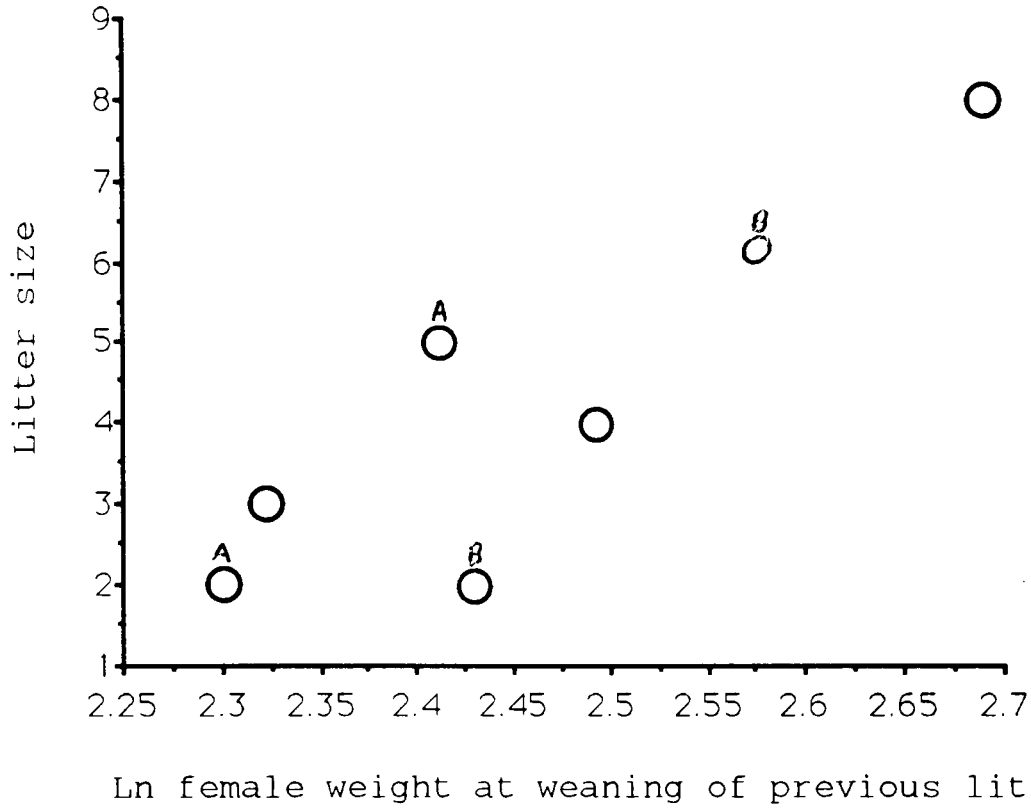

Figure 1. Relationship between the size of second or third litter and the weight of the mother at the weaning of her previous litter (data for female weight were log transformed to meet the requirements of a normal distribution). For the females, the data for both the second and third litters are included on this graph, but for statistical analysis (see text) single mean values were calculated for each female.

Letters are used to distinguish those points representing the weight of the same female after different litters.

Sex ratio.-- Of those offspring sexed on weaning, 57% (119 of 209 animals) were males (table 1). Although the sex ratio among weanlings did not differ significantly from 50%, there was a clear tendency towards male-bias, which agrees with the findings of Brambell (1935a), who gives an average sex ratio of 54% males for wild-caught animals throughout the year (n=1064). (Note that male-bias determined in nature may partly reflect behavioral differences, particularly in the spring: Crowcroft, 1957, Skarén, 1973, Pucek, 1959.) There were no significant differences in sex ratio between litters conceived in nature and those conceived in captivity.

Response to photoperiod.-- Thirteen males survived to give informative data (tables 3 and 4), and on the basis of testis and seminal vesicle weights, males subjected to each of the three photoperiod regimes attained sexual maturity. As regards those individuals kept singly (table 3), it is particularly striking that one individual each from regimes B (4 months LD) and C (2 months SD, 2 months LD) had a combined testis weight greater than 250 mg, comparable with males at the height of sexual development. Associated with testis growth, the skin over the testes became bare in some males, as recorded in wild-caught individuals (Searle, 1985a). One individual from regime C demonstrated regrowth of hair on this bare patch, also in line with animals in nature (J.B.S., personal observation).

Five animals were housed in groups (of 3 and 2 animals respectively, see table 4). Of the three males subjected to two months LD (regime A), one was of immature size and

reproductive condition, while the others clearly showed testis and seminal vesicle development. In the other regime (four months LD; regime B), one had barely begun maturation, whilst the other showed clear signs of adulthood, both in reproductive and body growth. Neither individual showed sexual development to the degree found in regime B animals housed singly (table 3). Inhibition of sexual maturation due to the proximity of another animal is a well-documented phenomenon in both sexes (Lee and McDonald, 1985; Spears and Clarke, 1986), and it is interesting that this may occur in the common shrew despite its normally solitary nature (Michielsen, 1966). All animals were in good health, and it is considered unlikely that individuals remained immature due to insufficient access to the food supply.

Although no significant differences were found between animals kept singly under regimes B and C, and individual variation was large, some trends should be noted (table 3). Seminal vesicle weight was similar in the two regimes (indicating similar androgen levels), but testis weight was higher on average under the B regime (4 months LD). In contrast, the animals under regime C (2 months SD, 2 months LD) had a more adult body to tail ratio, with a mean (± SE) of 1.87±0.13 (mean for regime B: 1.59±0.09). In both regimes there were males with adult testis weight and immature body proportions, suggesting sexual and physical maturation are to an extent independent processes, although in nature they usually occur concurrently. Pucek (1960) who clearly demonstrated that female common shrews of immature body

proportions can become sexually mature, made a similar postulation.

The lateral scent gland, which in nature only undergoes substantial development in the male (Searle, 1985a), became active in individuals of all three photoperiodic regimes, in parallel with body size and testis development.

Table 3.--The extent of sexual maturation in male shrews kept singly under different photoperiod regimes

Photo-period regime	Age[a]	Combined fresh weights(mg) Testes	Seminal vesicles	Body weight (g)	Body-to-tail ratio
B	191	282	105	9.27	1.51
(4 months	192	124	122	9.09	1.76
LD)	145[b]	156	82	5.64	1.49
Means:	176	187	103	8.00	1.59
C	189	255	100	8.46	1.60
(2 months	191	71	50	8.28	1.84
LD +	191	127	135	10.09	2.32
2 months	192	79	115	8.65	1.60
SD)	182	88	136	8.48	1.97
Means:	189	124	107	8.79	1.87

a Age in days from weaning.
b Animal died prematurely, and therefore received approximately five weeks less long photoperiod than the other two regime B animals (86 days, as opposed to 123 and 124 days). Its loss of condition is reflected by its low body weight.

Table 4.-- The extent of sexual maturation in male shrews kept in groups under different photoperiod regimes

Photo-period regime	Age[a]	Combined fresh weights(mg) Testes	Sem. vesicles	Body weight (g)	Body-to-tail ratio
A	112	117	118	8.6	--
(2 mo. LD)	112	75	29	6.5	--
	112	5	[b]	6.2	--
B	194	54[c]	12	7.8	1.82
(4 mo. LD)	194	20[d]	[b]	7.6	1.37

[a] Age in days from weaning.
[b] Too small to weigh accurately.
[c] Sperm and meiotic divisions observed in testes.
[d] No sperm in testes, no indications of meiosis.

Inherited characters.-- Breeding studies have demonstrated that alleles at the Mpi-1, Pgm-2 and Pgm-3 enzyme loci segregate in a Mendelian fashion in the common shrew (Searle, 1983, 1985b). We report here on segregation studies of other polymorphic features.

Various patterns of distribution of white fur have been noted on the body of the common shrew (Crowcroft, 1955). While white nape patches may be found in adult females as a result of damage during mating (Crowcroft, 1955) and a generalised white peppering is associated with old age (Searle, 1983), the incidence of white patches (commonly found on the ears, but also found in association with the feet, abdomen and tip of the tail-- Crowcroft, 1955) may have a genetic basis. If white spotting results from the expression of an allele at a particular locus, it would seem likely that the gene is analogous or homologous to the recessive, non-pleiotropic white-spotting gene of variable penetrance (s), found in several other mammals, e.g. guinea pigs (A.G. Searle, 1968).

The results from breeding studies are consistent with the interpretation that white spotting is controlled by an s-type gene. A number of offspring from crosses between white-spotted shrews did not display any form of white spotting (data in Searle, 1983), consistent with expression of a recessive trait. The fact that within an individual one ear may have white hair and the other not (Crowcroft, 1955) suggests that the white-spotting allele is not fully penetrant.

Breeding studies have also proved invaluable for cytogenetic analysis. In Britain, there are three karyotypic races of common shrew ('Oxford', 'Aberdeen' and 'Hermitage'),

each with a distinctive chromosomal complement. All of these races can be crossed in captivity and it has been demonstrated that litter sizes are similar to those derived from intra-racial crosses (Searle, 1984b). Furthermore, we have been able to bring Oxford - Aberdeen race hybrids to maturity by photoperiod manipulation (as described above), and to study their fertility (Chapter 3). Searle (1986) has also examined transmission of variant chromosomes from chromosomal heterozygotes. The results from wild-caught females are unreliable due to multiple paternity (which has itself been demonstrated with the help of this breeding study-- Appendix 1; see also Searle, 1990), but those from crosses in captivity can be used.

CONCLUSIONS

The success of this breeding program may be measured in terms of percentage of fertile crosses, survival of young to weaning, and postweaning survival. In all of these respects, and especially in terms of the number of young reared, the method has shown itself to be remarkably reliable. Other workers (M. Dodds-Smith, P.Stockley, personal communications) have successfully adopted the same protocol.

Altogether 50% of attempted crosses were fruitful. Collecting adult females during their first pregnancy rather than before enhances the success of subsequent captive crosses. Of those offspring known to be produced, 87% survived to weaning (excluding those young deliberately killed before weaning), but postweaning mortality was sometimes high, especially if the young were exposed to excessive disturbance.

The sample of animals subjected to photoperiodic manipulation was too small to allow thorough quantification of any of the observed effects. It seems certain, however, that prolonged periods of long days (16L:8D) stimulate sexual maturation (as indicated by testis size and seminal vesicle growth) in young common shrews. Under these conditions, sexual development may occur in the absence of body growth, and both may be inhibited when common shrews under long photoperiod are housed in groups. These observations deserve further study.

We have demonstrated that captive maintenance of the common shrew is feasible at all stages of the life cycle, and most of the technical obstacles to the establishment of a breeding colony have been removed. Shrews can be kept in standard laboratory cages for several months at a time, and early sexual maturation can be induced. The principal problems remain those of the frequency of feeding and the level of mortality, but both of these are likely to be overcome through a modification of existing techniques. The benefits of a permanent colony of common shrews for studies of behavior, traditional genetics and reproductive biology would be substantial. In particular, such a colony would permit a more detailed experimental approach than hitherto possible for the study of the phenomenal chromosomal variation in this species.

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CHAPTER 3

MEIOTIC STUDIES OF KARYOTYPICALLY HOMOZYGOUS AND HETEROZYGOUS MALE COMMON SHREWS

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ABSTRACT

Over a number of years, we have studied meiosis in male Robertsonian homozygotes and heterozygotes from the vicinity of the hybrid zone between the Oxford and Hermitage karyotypic races of common shrew in southern England. For the heterozygotes, most of the data derive from 'simple' heterozygotes which form trivalents at prophase/metaphase I, but some data are also available for 'complex' heterozygotes which form chains composed of more than three elements. Our general conclusion is that meiosis proceeds in a remarkably orderly fashion in Robertsonian heterozygotes.

At pachytene in simple Robertsonian heterozygotes, the three chromosomes which form the trivalent pair intimately along their length. Only rarely do the centromeric regions of the acrocentrics pair non-homologously to form a side arm. (By contrast, in the house mouse, such side arms are normal in Robertsonian heterozygotes.)

At diakinesis/metaphase I, regular trivalents can be observed in simple Robertsonian heterozygotes and quadrivalents in double Robertsonian heterozygotes with monobrachial homology. As at pachytene, univalence is unusual.

Chromosome counts at metaphase II indicate that anaphase I nondisjunction frequencies in male simple Robertsonian heterozygotes are no higher than those of Robertsonian homozygotes. Anaphase I nondisjunction leads to a reduction in fitness through production of aneuploid gametes and consequently aneuploid zygotes which are usually inviable. Clearly, male simple Robertsonian heterozygotes are not greatly unfit compared to homozygotes from this cause.

INTRODUCTION

Throughout the range of the common shrew, the species is subdivided on the basis of karyotype as a result of many different Robertsonian fusion mutations. Populations frequently differ in their diploid number and in their complement of metacentric chromosomes, although the *Nombre Fondamental* (NF) remains the same. In Britain, three chromosomal groupings are recognised (Searle 1984) and designated as 'karyotypic races', each possessing a different combination of Robertsonian fusion products specific to that race. In addition to these race-specific metacentrics, polymorphism for other fusions may give rise to local racial variants.

In southern Britain, the interface between the Oxford and Hermitage karyotypic races forms a complex hybrid zone and region of karyotypic polymorphism approximately 100km wide (Searle 1986a) running from the Thames estuary eastwards to the Severn river valley. Within the vicinity of this zone, a large number of karyotypes may be found, with combinations of the metacentric *ko*, specific to the Hermitage race, and *kq* and *no*, specific to the Oxford race (nomenclature of Halkka et al. 1974, modified by Fredga and Nawrin 1977). In addition, the local variant of the Oxford race possesses the metacentric *pr*, and the acrocentrics *n*, *q*, *p* and *r* are present on the Hermitage side. The zone is further complicated by the presence of the acrocentrics *k* and *o*.

Two different types of Robertsonian heterozygote are recognised. The 'simple heterozygote' possesses one

metacentric and two acrocentric homologues for one or more arm combinations. Synapsis during prophase I of meiosis is expected to result in the formation of a trivalent for each heterozygous arm combination. In some cases, however, an animal may possess two or more metacentrics that share only one arm in common (for example *ko* and *kq*), a monobrachial homology. In such a situation, synapsis is expected to result in the formation of a multivalent of four or more elements during prophase I of meiosis, and this type of individual is termed a 'complex heterozygote'. It should be noted that the common shrew has an XX/XY_1Y_2 sex chromosome constitution, and thus males are expected to form a 'sex trivalent' at prophase I of meiosis in addition to any autosomal multivalents.

Several studies of Robertsonian heterozygotes in mouse and man indicate that heterozygosity impairs fertility (Chandley 1984; Gropp and Winking 1981) and several mechanisms have been suggested to explain this. Firstly, the accuracy of homologous synapsis during meiotic prophase I is thought to affect the number of germ cells surviving to produce gametes, and this germ cell death may reduce fertility and gonad size. Secondly, nondisjunction at anaphase I may be increased and lead to the formation of unbalanced gametes, and postzygotic loss.

Although the mechanism is in debate, it would appear that in mammals a relationship exists between the degree of aberration at the time of pachytene pairing of homologues and the extent of germ cell death. Two mechanisms have been proposed to explain this relationship. The first is that association between unpaired regions of a heteromorphic

autosomal configuration and the sex bivalent in the male (or in the case of the common shrew, the sex trivalent) interferes with the obligatory inactivation of the X chromosome and results in germ cell death and sterility or subfertility (Forejt and Gregorová 1977). Whilst a good case can be made for this in many instances, it cannot explain subfertility in cases where no such association is observed, and is completely inapplicable to the female, where more germ cell death may also occur in karyotypic heterozygotes than in homozygotes (Mittwoch et al. 1981).

The observation that unpaired regions are found at pachytene in heterozygotes for chromosomal rearrangements gives rise to the second mechanism. Miklos (1974) proposed the existence of sites scattered throughout the chromosomes, which must be saturated through homologous pairing. Failure to do so will initiate a mechanism resulting in death of the cell. The time taken for cell death to occur would be proportional to the number of unsaturated sites, thus explaining the differences observed in severity and timing in similar chromosomal rearrangements. This mechanism has the advantage over that of Forejt in that it is equally applicable to both sexes. Burgoyne and Baker (1984) further discuss the applicability of the Miklos model. The fact remains that in mammals, chromosomal rearrangements generally have more severe consequences for males than for females, in respect to germ cell death, and it would therefore seem reasonable to assume that both the Forejt and the Miklos models operate in many cases. In the male shrew, we should therefore look both for an association between autosomal multivalents and the sex

trivalent, and also for accuracy of pairing within the multivalent.

The pattern of chromosome pairing at pachytene may also be relevant to the incidence of anaphase I nondisjunction in Robertsonian heterozygous common shrews. Thus, any minor inappropriate gene expression due to incomplete pairing, rather than being cell lethal, could result in disruption of the segregation process. Also, univalence at the pachytene stage (again, if not cell lethal due to inappropriate gene expression) could lead to random segregation at anaphase I. Alternatively, errors may not arise until orientation on the spindle, with the autosomal multivalents more prone to malorientation than a bivalent, merely because the spindle is better adapted to handle bivalents, but not the rarer multivalent configurations.

The current study combines the data from a number of recent studies of homozygotes and the different classes of heterozygote. It aims to help determine the extent of disruption of fertility in heterozygotes, and to help understand the evolution of the Oxford - Hermitage hybrid zone.

MATERIAL AND METHODS

All animals studied were males collected from the vicinity of the Oxford - Hermitage hybrid zone south of Oxford, England, with the exception of two individuals born in captivity from a wild caught pregnant mother, and subsequently matured under conditions of long photoperiod (chapter 2). All animals were killed by cervical dislocation, and mitotic

karyotypes were prepared from bone marrow using the method of Searle (1986a). These preparations were subsequently G-banded using a combined Trypsin/ASG method (Seabright 1972, Sumner et al. 1971, Searle 1986a), and karyotyped under oil immersion.

Air - dried preparations of testis material were made by the method of Evans et al. (1964), as modified by Searle (1986b). Anomalies were scored at diakinesis/metaphase I and anaphase I nondisjunction was estimated from metaphase II spreads. Cells were analysed at a magnification of 400X, with verification under oil at 1000X.

Pachytene data were gathered from surface spread material (Wallace and Searle 1990), silver stained and examined under the light microscope.

RESULTS

Pachytene

Wallace and Searle (1990) gathered data of 252 pachytene spreads from ten animals, and conclude that the pairing process at pachytene is orderly both in homozygotes and simple heterozygotes. No complex heterozygotes were available for study.

General features of the pachytene cell were examined, and the locations of four nucleolar organising regions determined (figure 1a). These were located distally on the arms *o*, *q*, *t*, and *u*, in accordance with silver staining (Olert and Schmid 1978; Halkka and Söderlund 1987), and the location of secondary constrictions (Searle 1983) in the mitotic karyotype. Although centromeres were frequently not visible, bivalents were identified on the basis of total length. The

sex trivalent paired 'straight through' with no discernible side arm, and no visible distinction between the autosomal and gonosomal arms (figure 1a). The unpaired region of the sex trivalent was frequently distinguishable, due to a hooked or wavy appearance, and the presence of excrescences (figure 1a).

In simple heterozygotes, the autosomal trivalent at pachytene was found to pair 'straight through' in the majority of cases, with side arms being present in only 36% of trivalents. In some of these (7% of trivalents) the arms were unpaired, with a minority (1% of trivalents) in the rare *trans* configuration (Moses et al. 1979). Other abnormalities were also rare; association between autosomal trivalents and the sex trivalent was estimated at between 2.8 and 7.5%. Univalence was estimated as only 4.4%, and no difference was detectable in this frequency between homozygous and heterozygous animals.

Diakinesis/metaphase I

Searle (1986b) presents data on diakinesis/ metaphase I for a total of 40 animals (16 Robertsonian homozygotes, 21 simple and 3 complex heterozygotes). A mean of 22.8 chiasmata were visible per spread, with some indication of differences in chiasma number between chromosomal race as well as between homozygotes and heterozygotes. The number of chiasmata per bivalent appears to positively correlate with length, with those arms commonly involved in Robertsonian fusions (*g* to *r*) tending to have one chiasma each, thus acrocentric bivalents usually have one chiasma.

The sex trivalent is clearly visible at this stage (figure 1b), with the Y_1 and one arm of the X (the 'true-X' segment) often distinctly heteropycnotic. The Y_1 appears to be terminally attached, and the attachment is scored as a single chiasma, with between one and three chiasmata joining the X and the Y_2.

Chain trivalents and quadrivalents respectively were observed in the simple and complex heterozygotes examined. These multivalents usually averaged one chiasma per chromosome arm, in either a distal or interstitial position.

The incidence of univalence scored at diakinesis/metaphase I may be calculated for homozygotes and for both categories of heterozygote (table 1), and the sum total univalence is found to be 4.5%. Although the frequency for simple heterozygotes (6.6%) is higher than that for homozygotes (2.5%), univalence in the complex heterozygotes is very low (0.7%), possibly due to the small sample of animals available for study. Much of the univalence in simple heterozygotes can be accounted for by separation of the X and Y_1 chromosomes.

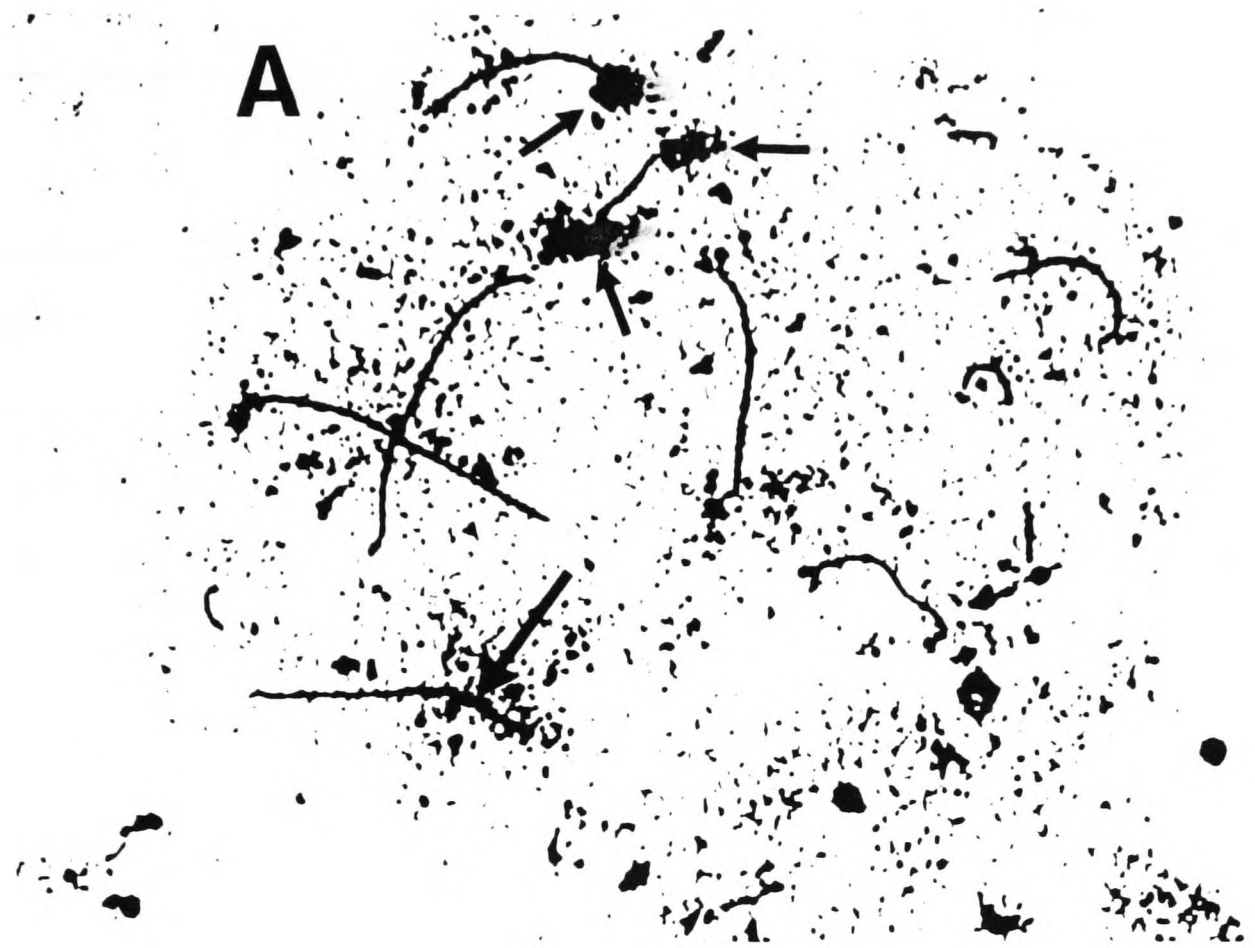
Comparison between the degree of univalence in single, double, and triple simple Robertsonian heterozygotes (i.e., individuals heterozygous for 1, 2, and 3 arm combinations with no complications of monobrachial homology) also indicates that the rise in univalence correlates with the number of trivalents present in the cell (table 2). Again, the increased univalence is primarily due to the presence of a univalent Y_1.

Figure 1.

(a) Pachytene spread of a Hermitage race individual (2n=25, homozygous metacentric for arm combinations *jl* and *ko*, homozygous acrocentric for arms *n*, *p*, *q* and *r*) after silver staining. Note nucleolar material associated with chromosome arms *t*, *u* and *o* (short arrows), and excrescences around the unpaired region of the sex trivalent (long arrow).

(b) Diakinesis spread of an Aberdeen race individual (2n=21, homozygous metacentric for arm combinations *jl*, *ko*, *np* and *qr*). Note sex trivalent (arrow).

(c) Metaphase II spread of an Oxford race individual in which arm combinations *jl*, *kq*, *no* and *pr* are all present in fully metacentric form. The X chromosome is marked by an arrow.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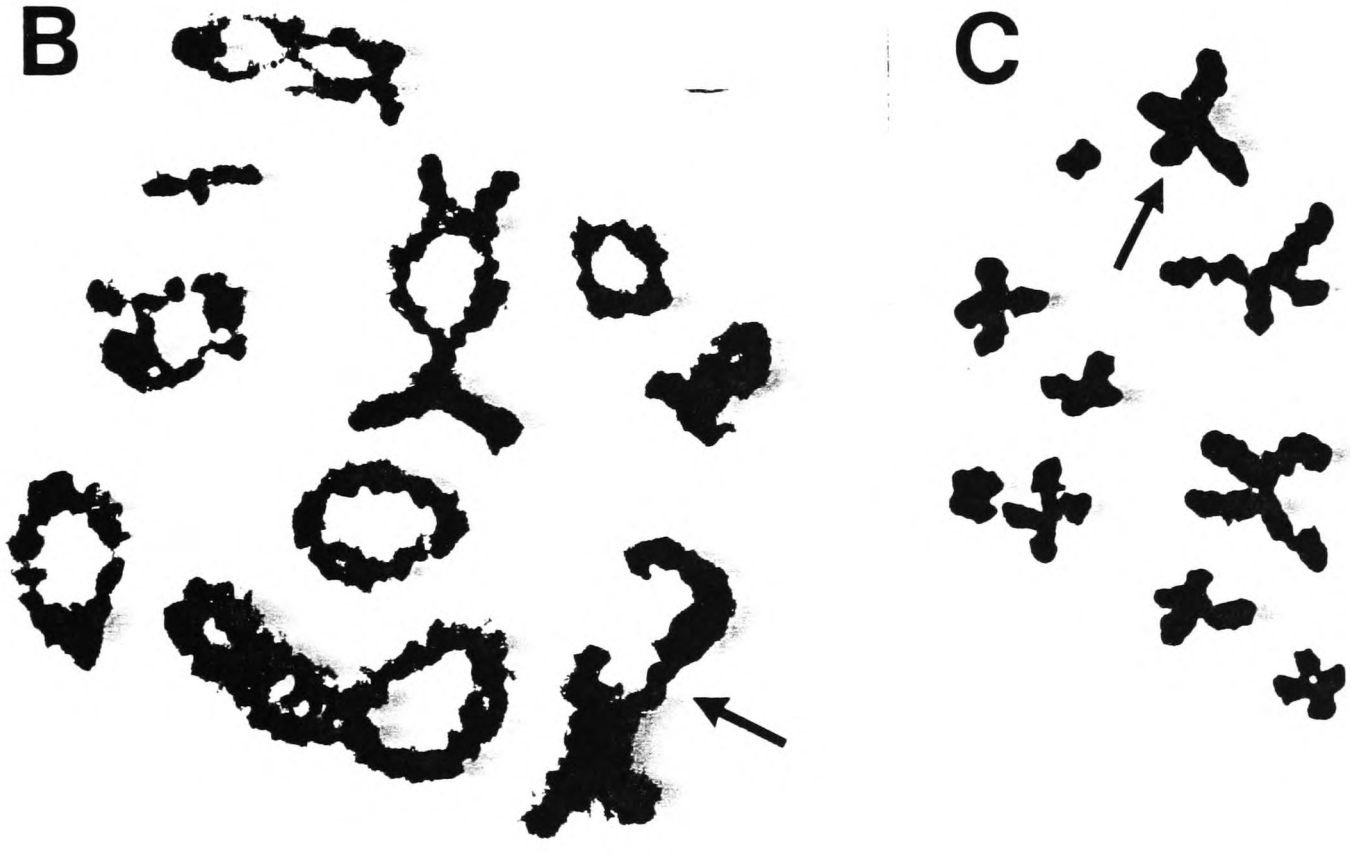
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Table 1. Univalence at diakinesis/metaphase I in individuals of different karyotype

	No. Animals (spreads)	Univalence			
		X/Y_1	chain	other	unknown
Homozygote[1]	16 (800)	15	4	-	1
Simple Het.	21(1050)	32	5	21	11
Complex Het.	3 (150)	1	0	0	0

1 Includes six individuals not collected from the Oxford - Hermitage karyotypic hybrid zone.

Table 2. Univalence in single, double, and triple simple heterozygotes at diakinesis/metaphase I

	No.Animals	Univalence			
	(spreads)	X/Y_1	Trivalent	Other	Total
Single	9(450)	2.2-2.9%	1.6-2.2%	0.4-1.1%	4.9%
Double	5(250)	4.4-5.2%	1.6-2.4%	0.4-1.2%	7.2%
Triple	2(100)	4.0%	3.0%	0.0%	7.0%

Metaphase II

At metaphase II, all elements appear diffuse, with chromatids frequently separated. One arm of the X (the 'true X' segment) and the Y_1 frequently appear heteropycnotic in X or Y bearing spreads respectively (figure 1c). Anaphase I nondisjunction rates can be calculated from the incidence of hyperploidy (table 3).

With a total dataset of 20 animals and 366 metaphase spreads, the incidence of nondisjunction is calculated as 2.3% for homozygotes, 2.1% for single simple Robertsonian heterozygotes, and 0% for double simple heterozygotes. Therefore, heterozygosity causes no detectable elevation in the rate of anaphase I nondisjunction in these animals.

Table 3. Chromosome counts at metaphase II in individuals of different karyotype

	No. Animals (spreads)	No. Elements				Nondisjunction (%)
		<18	18	19	20	21	
Homozygotes[1]	8 (88)	0	2	13	72	1	2.3%
Single simple heterozygotes[2]	9(189)	0	6	19	162	2	2.1%
Double simple heterozygotes[3]	3 (89)	1	2	3	83	0	0%

[1] Includes one animal bred in captivity (A2111), and one animal from the Aberdeen race (2121). The complete dataset for homozygotes, given in the form (n=18,19,20,21) is: 2092(0,0,3,0) 2093(0,1,11,0) 2094(0,0,10,0) A2111(2,3,2,0) 2121(0,2,11,0) 2395(0,0,3,0) 2417(0,0,1,0) 2420(0,7,31,1).

[2] Includes one animal from the Hermitage race, two from the Aberdeen race (one bred in captivity), and three Oxford race animals not from the region of the Oxford - Hermitage hybrid zone. In addition to the data of Searle (1986b) new data are: 2065(0,2,2,0) 2076(1,5,21,1) 2077(1,0,16,0) 2091(1,1,16,0) B2118(0,0,1,0).

[3] Data of Searle (1986b), plus 2079(1,2,9,0).

The data in these footnotes is tabulated in Appendix B, table 4.

DISCUSSION

It is clear that although substantial variation was found between individual common shrews, meiosis in homozygous and simple heterozygous males is orderly, with only a very low level of irregularity. Pairing at pachytene appears to be precise, with nonhomologous pairing and the formation of side arms rare (side arms are very small if present). Association between the sex trivalent and autosomal trivalents was uncommon, and can probably be explained as random orientation of synaptonemal complexes during preparation. The incidence of univalence at pachytene is very low, and agrees closely with the overall level observed at diakinesis/metaphase I. At diakinesis/metaphase I, however, there is an indication that Y_1 univalence, albeit rare, increases with heterozygosity.

Given this general regularity in chromosome behaviour in male simple heterozygotes, it is not surprising that they show no increase in anaphase I nondisjunction over homozygotes. Nor is there any substantial increase in the incidence of germ cell death (Garagna et al. 1989; Wallace et al. 1991), as may be predicted from both the Forejt and Miklos models.

For the complex heterozygous males from the Oxford - Hermitage hybrid zone, only diakinesis/metaphase I data are available. The individuals examined formed chain quadrivalents with little irregularity. Garagna et al. (1989) found that spermatogenesis proceeds reasonably well in these individuals, although there appear to be indications of a greater degree of germ cell death than in male homozygotes or simple heterozygotes. The same may be said for male laboratory-bred hybrids between the Oxford and Aberdeen karyotypic races

(chapter 4), which formed a regular chain VII configuration at prophase I (*r - rp - pn - no - ok - kq - q*). In these individuals, abnormalities were observed at pachytene and there may be a higher frequency of anaphase I nondisjunction than in homozygous or simple heterozygous males.

It has been assumed that the maintenance of the Oxford - Hermitage hybrid zone, and other karyotypic hybrid zones like it, is due to some mechanism of heterozygote disadvantage. In the case of a zone separating two races of shrew differing in Robertsonian metacentrics, the assumption is that the unfitness of the heterozygotes is attributable to meiotic irregularity arising from Robertsonian heterozygosity. If this is true, then our data suggest that hybrid unfitness must either be attributed to heterozygous females (but see Searle 1990), or complex heterozygous males. There is no evidence that males that are simple Robertsonian heterozygotes suffer reduced fitness relative to homozygotes.

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NOTE IN PROOF

Common shrews that are karyotypic homozygotes and simple Robertsonian heterozygotes have recently been studied at pachytene under the electron microscope (Fredga and Ashley 1990; Borodin 1991), affording greater resolution than we could obtain at the light microscopic level. These EM studies show that instead of end-to-end pairing between the X and Y_1 chromosomes, as we suggested (see Wallace and Searle 1990), the Y_1 becomes paired with the X along its whole length. We have now been able to see the same phenomenon under the light microscope (B M N Wallace, personal observation).

Borodin (1991) examined autosomal trivalents in simple Robertsonian heterozygotes and notes that these configurations are fully paired throughout pachytene; this accords well with our results (see Wallace and Searle 1990). However, Borodin (1991) found that pachytene trivalents in Siberian shrews have a substantial side-arm due to extensive non-homologous pairing between the centromeric regions of the acrocentrics. This contrasts strongly with trivalents in British shrews which usually have no side-arms. It is most interesting that there should be such differences in morphology of the trivalent between Robertsonian heterozygotes from different geographical regions (and different inter-racial hybrid zones). We are grateful to Karl Fredga and Pavel Borodin for discussing these issues.

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CHAPTER 4

MALE COMMON SHREWS (*SOREX ARANEUS*) WITH LONG MEIOTIC CHAIN CONFIGURATIONS CAN BE FERTILE: IMPLICATIONS FOR CHROMOSOMAL MODELS OF SPECIATION

S.J. Mercer, B.M.N. Wallace, and J.B. Searle

ABSTRACT. Two chromosomal races of common shrew (*Sorex araneus*) were crossed in captivity to generate chain VII-forming complex Robertsonian heterozygotes. Meiosis and gametogenesis were studied in three male hybrids. Regular chain VII configurations were observed at both pachytene and diakinesis/metaphase I, although in many pachytene spreads the chain configuration was incomplete; the basis of this peculiarity is unknown. From metaphase II counts, the frequency of anaphase I nondisjunction in the complex heterozygotes was estimated to be 13%. There was 22% greater germ cell death in the chain VII-forming complex heterozygotes than in controls, but this is unlikely to have greatly influenced their capacity to sire offspring. Thus, the fecundity of these complex heterozygous common shrews is likely to have been only slightly reduced relative to homozygous or simple heterozygous shrews. These results call into question the generality of speciation models based on presumed sterility of complex heterozygotes.

Over the last 25 years there has been a lively discussion regarding the importance of chromosomal rearrangements in speciation. Such rearrangements have been implicated in speciation (White, 1968), firstly because closely related species often differ in karyotype, and secondly because heterozygotes are expected to suffer a higher incidence of meiotic aberrations than homozygotes, leading to reduced fertility. If chromosomal heterozygosity induces such a fertility reduction in hybrids between subspecies or races

which differ in karyotype, then it may augment the transition of such subspecific forms into species. If such hybrids can be seen to be sterile purely as a result of their chromosomal heterozygosity, this would imply that a process of 'chromosomal speciation' has already occurred.

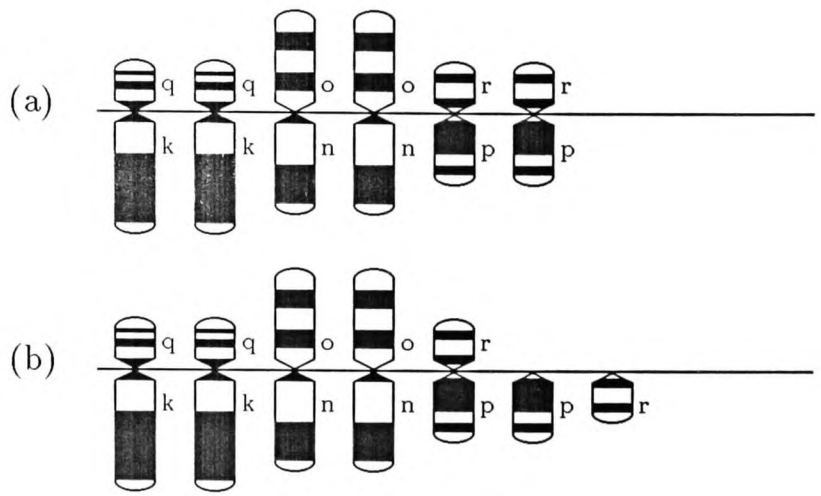
In mammals, any karyotypic difference between closely related species, or between races within species, often involves Robertsonian rearrangements (e.g. Baker and Bickham, 1986; Searle, in press). In the simplest case, the karyotype of one race differs from another by occurrence of a metacentric instead of two acrocentrics. Hybrids between such races may be called single 'simple' Robertsonian heterozygotes and would be expected to form a trivalent at meiosis I (figure 1). In the 1970's, studies on house mice suggested that such heterozygotes may suffer much reduced fertility relative to homozygotes, due to increased frequencies of anaphase I nondisjunction (males: Cattanach and Moseley, 1973; females: Winking and Gropp, 1976), supporting the idea that chromosomes can be important in speciation (White, 1978). The early mouse studies were, however, mostly based on individuals with a part-feral, part-laboratory strain genetic background (reviewed by Searle, 1988a); it has become apparent that individuals with such a hybrid genome are more prone to anaphase I nondisjunction than Robertsonian heterozygotes from nature (Winking, 1986). Wild-caught simple Robertsonian heterozygotes of several species of mammal have been shown to have very low frequencies of anaphase I nondisjunction (common shrew: Searle, 1984 [females], Searle, 1986 [males]; male house mice: Winking, 1986; male marsh rats: Nachman, 1990). To

date, only wild individuals heterozygous for one to three arm combinations have been examined (see figure 1); multiple Robertsonian heterozygotes heterozygous for many arm combinations may be considerably less fertile (compare Tettenborn and Gropp, 1970 with Cattanach and Moseley, 1973).

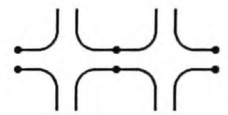
Another form of Robertsonian heterozygote is one in which the karyotype includes at least two different metacentrics, some of which share a common chromosome arm. These 'complex' Robertsonian heterozygotes are expected to form ring or long chain configurations at meiosis I (figure 1). Again, early studies on the house mouse indicated low fertility of complex heterozygotes relative to homozygotes, due to both an increased frequency of anaphase I nondisjunction and/or substantial germ cell death (which, in the male, could lead to azoospermia), as reviewed by Gropp et al. (1982). These studies were partly based on feral/laboratory strain hybrids, but also included inter-racial hybrids with an entirely feral genetic background. Subsequent studies on inter-racial hybrids in rats (Baverstock et al. 1983) and lemurs (Rumpler et al. 1985), supported the finding in mice that complex heterozygotes are either sterile or have very low fertility. Thus, it was reasonable for Capanna (1982) and Baker and Bickham (1986) to suggest that mammalian Robertsonian races which produce complex heterozygotes on hybridization are likely to be genetically isolated from each other and hence should really be considered separate species. More recent results on the fertility of complex heterozygotes cast doubt on the generality of this latest hypothesis of 'chromosomal speciation'. Chain IV-forming complex Robertsonian

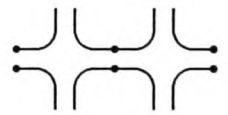
Figure 1. Idiograms and metaphase I configurations to illustrate the difference between 'simple' Robertsonian heterozygotes (which form one or more chain trivalent configurations at meiosis I) and 'complex' Robertsonian heterozygotes (which form chain configurations of 4 or more elements, or ring configurations). These idiograms are based on karyotypes of shrews from the Oxford and Aberdeen races (see Searle, 1988b). The following are illustrated: (a) a homozygote with a fully metacentric Oxford race karyotype, (b) a single simple heterozygote (Oxford race), (c) a multiple simple heterozygote (more precisely, a triple heterozygote: Oxford race), (d) a ring-forming complex heterozygote (more precisely, a ring VI-forming heterozygote: Oxford-Aberdeen race hybrid), and (e) a chain-forming complex heterozygote (more precisely, a chain VII-forming heterozygote: Oxford-Aberdeen race hybrid).

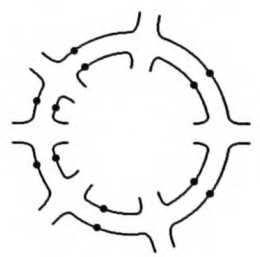
Figure 1. **Variable chromosomes** **Multivalents (metaphase I)**

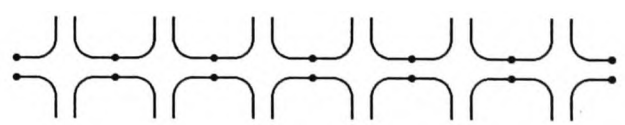


(a) none

(b)  trivalent

(c) 3  trivalents

(d)  ring VI

(e)  chain VII

heterozygotes in wild pigs (males and females) have normal litter size (Troshina et al. 1985), while in marsh rats, males with this karyotype have a low frequency of anaphase I nondisjunction (Nachman, 1990). In addition, the fertility of five wild-caught common shrews that were chain IV-forming complex heterozygotes has been examined. The four males had an increased incidence of germ cell death, but not apparently to a degree that would have caused reduced fecundity (Garagna et al. 1989b). The single female had eight fetuses, with no ovulations wasted as a result of anaphase I nondisjunction (Searle, 1990a).

These data argue that, for at least some species of mammal, complex Robertsonian heterozygotes which form short chains at meiosis I may not be particularly infertile in nature. In order to establish the extent of 'chromosomal speciation', it is important to further examine the degree to which complex heterozygosity is associated with sterility. In particular, there is a need to examine complex heterozygotes which form rings or *long* chains at meiosis I. In this paper, we report meiotic and histological data on male complex heterozygous common shrews which form chain VII configurations.

MATERIALS AND METHODS

Adult common shrews of the 'Aberdeen' and 'Oxford' chromosomal races (Searle 1988b) were collected in England from Frost Street (Somerset) and Salcey Forest (Northamptonshire) respectively, during May 1988. Crosses between Aberdeen race males and Oxford race females resulted

in hybrid offspring with a karyotype including metacentrics *ko, kq, no, np,* and *pr* and the acrocentrics *q* and *r* (nomenclature: Searle et al. 1991). These offspring were complex Robertsonian heterozygotes expected to form a chain VII configuration at meiosis I (see figure 1e). They were rapidly matured over two months under artificial photoperiod (see chapter 2), such that three adult males became available for study. Two were siblings (D2131, E2131), the other (B2111) had different parents.

Meiotic preparations were made from one testis by the air-drying method of Evans et al. (1964), as modified by Searle (1986), for chromosome counts at diakinesis and metaphase II. Surface spread preparations were also made (Hultén et al. 1985; Wallace and Searle, 1990), so that pachytene nuclei could be screened under the light microscope after silver staining.

The combined testis weights of all three adult male hybrids (D2131: 102.4 mg, E2131: 89.9 mg, B2111: 144.5 mg), were typical of sexually mature individuals early in the natural breeding season (Brambell, 1935). Standard 7 μm histological sections of the second testis were stained with haematoxylin and eosin, for general examination (Searle, 1988a), and by the periodic acid Schiff reaction (counterstained by haematoxylin) for germ cell counts (Drury and Wallington, 1980). For each animal, 50 seminiferous tubules in one cross-section of the haematoxylin and eosin-stained preparations were scored for presence/absence of spermatids or spermatozoa (Searle, 1988a). From the periodic acid Schiff-stained preparations, round spermatids and primary

spermatocytes were counted in seminiferous tubule cross-sections at stages II, VI and VIII of the ten-stage seminiferous epithelium cycle (Garagna et al. 1989a), and the Abercrombie correction applied (Garagna et al. 1989b). These are three of the most easily recognised stages, spaced over the epithelial cycle, but the spermatids are elongate at stage VIII and therefore not appropriate to score. A ratio of primary spermatocytes to round spermatids close to 1:4 may be expected in an animal with normal spermatogenesis, whereas germ cell death during meiosis or early spermiogenesis would result in relatively fewer spermatids. The spermatocyte: spermatid ratios for the hybrids were compared with three wild-caught controls. Two were homozygotes (shrew 2088: *pr, k,n,o,q*; 2089: *k,n,o,p,q,r*) and one was a double simple heterozygote (2090: *n/o, p/r, k,q*); all controls and hybrids were homozygous metacentric for chromosome *j1* (nomenclature after Searle et al. 1991; see also figure 1). For all individuals, five tubule cross-sections were examined for each of the three stages of the epithelial cycle scored.

RESULTS

Pachytene

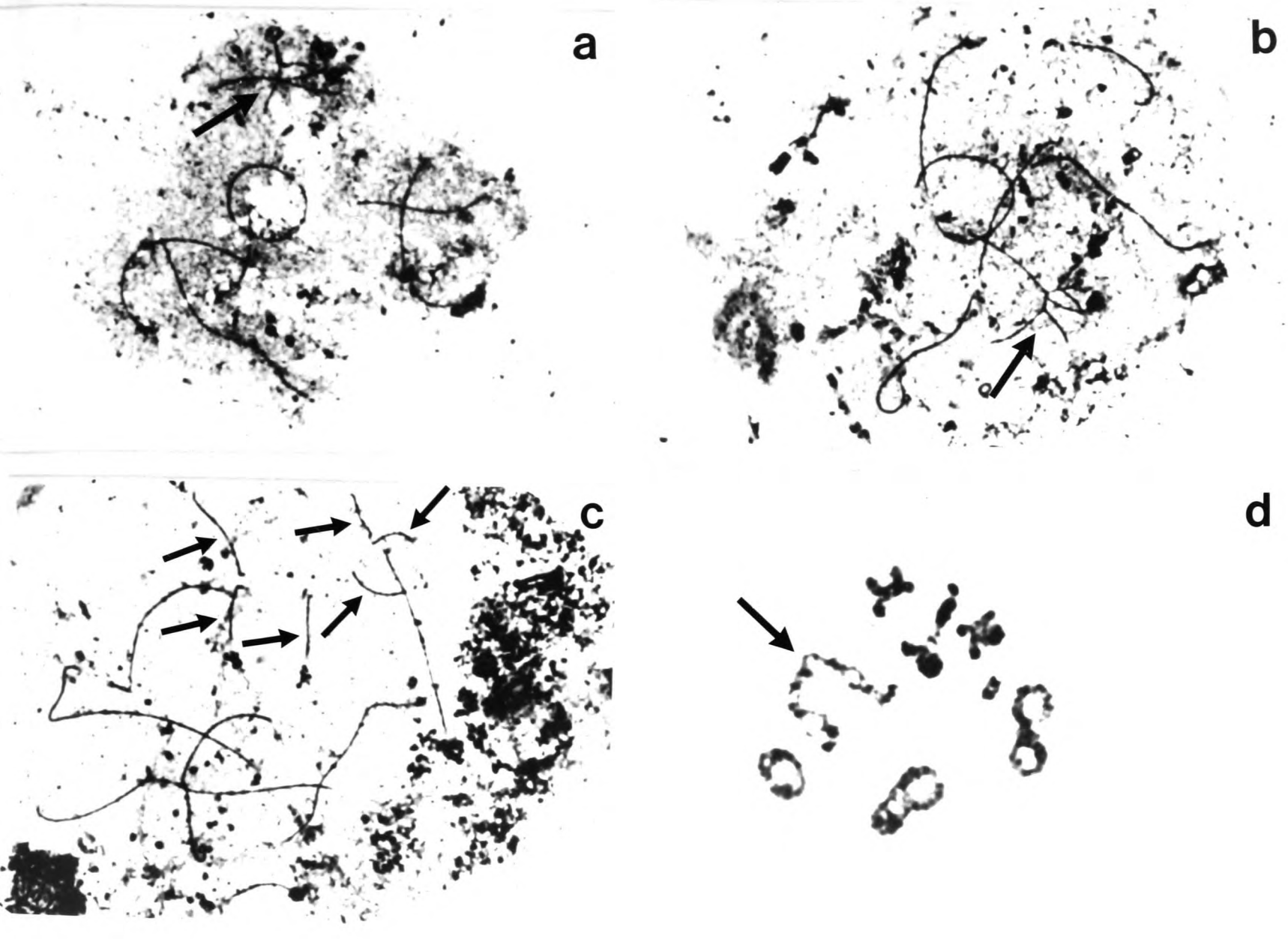
Synaptonemal complexes were examined under the light microscope in a total of 95 silver-stained pachytene spreads from the complex Robertsonian heterozygotes. In all cases, the bivalents and sex trivalent (XY_1Y_2) appeared to be fully synapsed, as recorded previously in homozygotes and simple heterozygotes (Wallace and Searle, 1990).

The pachytene behaviour of the chain VII configuration was more variable (table I). When complete, this generally appeared as a stellate synaptonemal complex with six arms (figure 2a). The length of these arms and the location of nucleolar material was consistent with the mitotic characteristics of the chromosomes concerned (see Wallace and Searle, 1990). Pairing appeared complete along the length of the majority of the chromosomes concerned (figure 2). However, there is less certainty about the pattern of pairing in centromeric regions. Irregularities at the 'hub' of the configuration (figure 2a, b) may reflect 'twisting' or 'stretching' during surface-spreading. Alternatively or additionally, there may be a degree of unpairing or nonhomologous synapsis. In 6% of complete configurations an additional short 'arm' was seen to radiate from the hub of the configuration (figure 2b). This most likely represents either the unpaired centromeric region of one of the acrocentrics (*q* or *r*), or extensive nonhomologous synapsis between the centromeric regions of these acrocentrics (Borodin 1991 records such nonhomologous synapsis in trivalents). Thirteen percent of the complete chain VII configurations examined overlapped the sex trivalent, while in 52% of the same cells there was overlap between the sex trivalent and at least one bivalent (table I).

Only a minority of spreads scored (33%: table I) displayed complete chains of the type described above. In the majority of spreads (56%), the chain consisted of less than six 'arms', with the missing lengths of synaptonemal complex (each equivalent to an arm of complete chain) dispersed around

Figure 2. Meiotic spreads from complex heterozygous shrews that are expected to form one chain of seven chromosomes (large arrow), and seven other configurations at prophase/metaphase I. (a) Pachytene spread with complete chain VII configuration which has a stellate appearance, with six arms radiating. (b) Pachytene spread in which stellate configuration has a seventh short 'arm'. (c) Pachytene spread with no chain configuration, instead six isolated regions of synaptonemal complex (small arrows). (d) Diakinesis spread.

Figure 3. Section through a seminiferous tubule at stage V of the epithelium cycle (Garagna et al. 1989a) from a chain VII-forming complex heterozygous shrew. Note large numbers of round spermatids (St) and fully-formed spermatozoa (Sz).



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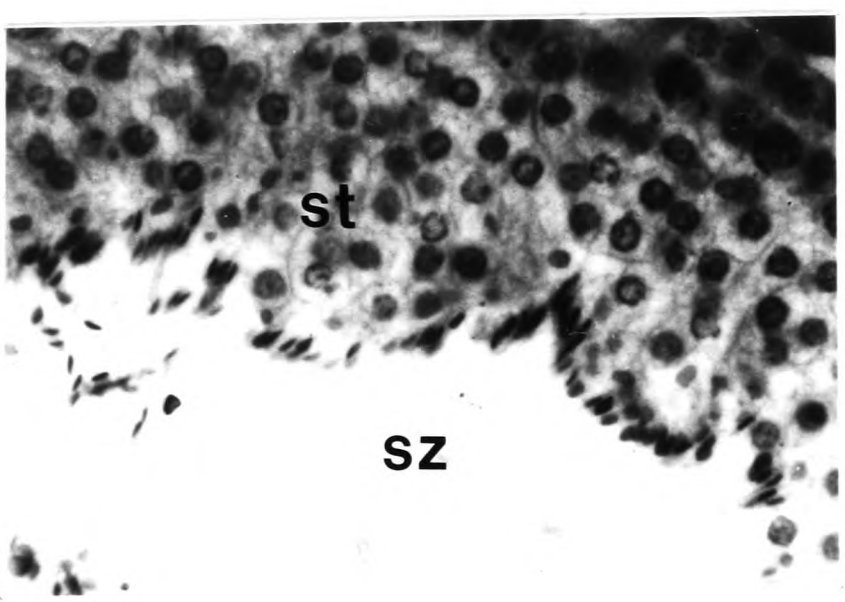

3

the cell. In the remaining 12% of spreads, none of the six 'arms' were attached to each other (figure 2c).

From our light microscope preparations, it was not possible to establish pachytene substages with any precision (see Wallace and Searle, 1990). On the basis of electron microscope studies, however, there was the expectation that 'excrescences' associated with the X axis would be more obvious later in pachytene (Fredga and Ashley, 1990). In our studies of chain VII-forming complex heterozygotes, there was no significant association between presence of excrescences and degree of completeness of the chain configuration.

Diakinesis/metaphase I and metaphase II

From the air-dried preparations, there were few indications of abnormality at diakinesis and metaphase I in the complex heterozygous shrews (figure 2d). There was a low frequency of univalence, associated with incompleteness of the chain VII or sex trivalent configurations (table II). Contact between these two configurations was limited to 5.8% of spreads.

It is difficult to obtain large numbers of scorable metaphase II spreads in the common shrew (Searle, 1986; chapter 1); only 16 spreads could be analysed from the three hybrid males. Of these, nine were euploid (20 chromosome arms), six were hypoploid (five with 19 arms, one with 18) and one was hyperploid (21 arms), giving an estimated anaphase I nondisjunction frequency of 13% (twice the hyperploid frequency: Cattanach and Moseley, 1973; Searle, 1986).

Table I. Pachytene pairing in surface spread nuclei from chain VII-forming complex heterozygous male shrews

Shrew	Total number of spreads	Number of spreads				
		Complete chain	Incomplete chain	No chain	Sex triv-chain contact[a]	Sex triv-bivalent contact[a]
B2111	11	3 (27%)	7 (64%)	1 (9%)	2 (67%)	1 (33%)
D2131	53	22 (42%)	26 (49%)	5 (9%)	2 (9%)	13 (59%)[b]
E2131	31	6 (19%)	20 (65%)	5 (16%)	0 (0%)	2 (33%)[c]
Total	95	31 (33%)	53 (56%)	11 (12%)	4 (13%)	16 (52%)

[a] This was only scored when a complete chain was present. There was no indication of any greater contact between the sex trivalent and elements expected in the chain, when the chain was incomplete or absent.

[b] In three spreads the sex trivalent overlapped two bivalents.

[c] In one spread the sex trivalent overlapped two bivalents.

Table II. Number of spreads with anomalies at diakinesis/metaphase I in chain VII-forming complex heterozygous male shrews

Shrew	Total number of spreads scored	Incomplete sex trivalent[a]	Incomplete chain[a]	Sex trivalent-chain contact
B2111	82	1 (1.2%)	1 (1.2%)	6 (7.3%)
D2131[b]	107	3 (2.8%)	3 (2.8%)	6 (5.6%)
E2131	124	2 (1.6%)	1 (0.8%)	6 (4.8%)
Total	313	6 (1.9%)	5 (1.6%)	18 (5.8%)

[a] These were associated with the presence of univalents (Y_1 or one acrocentric autosome [*p* or *r*], respectively).

[b] In one spread, two univalents were present in the place of a small autosomal bivalent (probably *tu*).

Germ cell death

Gross examination of seminiferous tubules in the chain VII-forming complex heterozygotes indicated no major disruption of spermatogenesis, with spermatids or spermatozoa visible in all tubules (figure 3). An impression was gained, however, that there were fewer spermatozoa in the seminiferous tubule cross-sections at epithelial stage V (Garagna et al. 1989a), compared to the controls. In line with this observation, it was found that the number of round spermatids at stages II and VI was reduced in comparison with homozygous and simple heterozygous controls (table III). The ratio of primary spermatocytes to round spermatids averaged 1:3.1 (compared with 1:4.0 averaged over the three controls), and therefore there was 22% greater germ cell death in the hybrids than the controls during meiosis and early spermiogenesis.

Table III. Mean numbers of spermatocytes and spermatids per cross-section of seminiferous tubule (after Abercrombie correction: Garagna et al. 1989b) at different epithelial stages (Garagna et al. 1989a), in chain VII-forming complex heterozygous hybrids and homozygous and simple heterozygous controls

Karyotypic group	Shrew	Primary spermatocytes II	VI	VIII	Round spermatids II	VI	S'cyte:s'tid ratio[a]
Control	2088	29.0	24.6	32.1	123.2	110.0	1:4.1
	2089	37.2	40.8	38.5	160.6	134.2	1:3.8
	2090	23.5	24.3	26.1	100.6	105.0	1:4.2
Hybrid	B2111	24.3	25.2	25.8	91.0	66.5	1:3.1
	D2131	24.6	18.1	18.5	66.3	55.0	1:3.0
	E2131	21.6	22.6	16.3	55.4	74.1	1:3.2

[a] Calculated from the means of all measurements taken for primary spermatocytes and round spermatids, respectively.

DISCUSSION

The greatest degree of abnormality observed in the chain VII-forming complex heterozygous shrews was at pachytene. In the majority of spreads, the chain VII configuration was incomplete, with some or all of the six chromosome arms that make up this configuration individually represented by isolated synaptonemal complexes. We can think of three explanations for these incomplete configurations.

Firstly, artifactual breakage of the chain VII synaptonemal complex could occur during surface-spreading. With this explanation, the difference between spreads with complete and incomplete chain configurations may have no biological meaning.

Secondly, the incompleteness of the chain configuration may be an indication of asynapsis in centromeric regions within the chain. Thus, in all spreads examined it would appear that the homologues for each of the individual chromosome arms (*k, n, o, p, q, r*) are largely synapsed, but the arms are not always attached to each other as would be expected, given that the chain configuration includes the metacentrics *ko, kq, no, np* and *pr*. If the centromeric regions of these metacentrics are unpaired, then the lateral elements representing these regions may have become stretched (perhaps partly as a technical artifact) and poorly visible, such that only the 'isolated' chromosome arms are easily seen at the light microscope level (see figure 2a in Ratomponirina et al. 1988).

The implication of this second explanation is that there is a great deal of asynapsis at pachytene in the complex heterozygous shrews. According to the hypothesis of Miklos (1974), extended by Burgoyne and Baker (1984), such asynapsis would be likely to lead to germ cell death. In contrast, Mahadevaiah et al. (1990) suggest that such asynapsis in a pachytene cell may indicate that the cell is already dying. While there is greater germ cell death in the chain VII-forming complex heterozygotes than in homozygotes and simple heterozygotes (which show very little pachytene asynapsis: Wallace and Searle, 1990), the degree of cell loss during meiosis and early spermiogenesis is not excessive. Clearly, those cells that were apparently showing asynapsis were not, in every case, already dying, as might have been expected from the hypothesis of Mahadevaiah et al. Unfortunately, the pachytene spreads could not be substaged accurately; thus, a possible explanation for the discrepancy between our results and the Miklos hypothesis may be that the majority of cells showing asynapsis become fully synapsed late in pachytene, and that such late synapsis is compatible with cell-survival. Late synapsis may include nonhomologous pairing of centromeric regions of the metacentrics and possibly the pairing of the centromeric regions of the acrocentrics *q* and *r*.

If there is such complete synapsis by a 'critical' late stage of pachytene, then the low frequency of contact between the chain configuration and sex trivalent in the hybrid shrews could also be explained. Asynapsed regions, particularly the end elements of chain configurations (Gropp et al. 1982), have a tendency to associate, and apparently interact, with the sex

chromosomes (Forejt, 1984). At both pachytene and diakinesis/metaphase I in our material, a relatively small proportion of spreads showed contact between the sex trivalent and chain VII (13% and 6%, respectively). The pachytene data can be seen in the context of overlap between the sex trivalent and at least one of the six autosomal bivalents, occurring in 52% of spreads. Given the large size of both the sex trivalent and the chain configuration, such a degree of overlap may be expected to arise as a result of chance juxtaposition during spreading (see Wallace and Searle, 1990); there is no evidence of physiologically-important interactions between the sex trivalent and chain VII configurations.

A third explanation for the incompleteness of the chain VII configuration at pachytene is that the dispersion of synaptonemal complex results from breakage *in vivo*. Fragmentation of synaptonemal complexes has been recorded in infertile men, and is thought to be associated with degeneration of spermatocytes (Vidal et al. 1982; Speed and Chandley, 1990). However, if all the pachytene cells we scored with an incomplete chain configuration were destined for degeneration, a greater degree of germ cell death should have been recorded. Clearly, an analysis of complex heterozygous shrews at the electron microscope level (Fredga and Ashley, 1990; Borodin, 1991) is required to decide between these three explanations for the incomplete chain configurations at pachytene.

Implications for chromosomal models of speciation

No attempt was made to mate the chain VII-forming complex Robertsonian heterozygotes described in this paper, and so their fertility must be inferred. There was greater germ cell death during meiosis and spermiogenesis in these male hybrid shrews than in the homozygous and simple heterozygous controls. Spermatids and spermatozoa were produced in substantial numbers, however, and it is unlikely that a 22% reduction in germ cell numbers would be sufficient to influence fertility of the hybrids (although sperm requirements may be quite large in shrews because of their promiscuous mating system: Searle, 1990b). Certainly, in male house mice a 35% reduction in germ cell numbers (between the pachytene and round spermatid stages of development) does not substantially reduce the ability to fertilise (Gropp et al. 1982) and a 90% reduction in numbers of spermatozoa is generally necessary to induce sterility (A. G. Searle and Beechey, 1974).

There are few data on the contribution of anaphase I nondisjunction to fertility impairment in the chain VII-complex heterozygous shrews, but the majority of metaphase II spreads scored were euploid (56%) and probably the majority of the hypoploid spreads (38%) were balanced spermatocytes from which chromosomes were lost during preparation as a result of cell breakage (Cattanach and Moseley, 1973). Thus, while it is likely that anaphase I nondisjunction is higher in the complex heterozygotes than in homozygotes or simple heterozygotes (chapter 3), they are a long way from being made sterile by this source of meiotic aberration.

In the common shrew, therefore, there is clear evidence that complex heterozygous males with long meiotic chain configurations need not be sterile. This has direct implications for at least one hybrid zone. In Siberia, there is hybridization between the Tomsk and Novosibirsk chromosomal races of common shrew, such that chain VII- and chain IX-forming complex heterozygotes are produced (Volobouev, 1983; Aniskin and Lukianova, 1989). Previously, it would have been reasonable to suggest that these hybrids are sterile and that the two races have become reproductively isolated as a result of 'chromosomal speciation'. Our results make this interpretation questionable.

Overall, the data available on mammals does suggest that complex heterozygotes (particularly those with long meiotic chains) tend to be less fertile than simple heterozygotes or homozygotes (Searle, in press). Complex heterozygotes can undoubtedly influence the structure of hybrid zones between chromosomal races (Searle, 1988a; Searle, in press), but further fertility studies are urged to fully assess the generality of the 'chromosomal speciation' models of Capanna (1982) and Baker and Bickham (1986). While studies of laboratory hybrids of house mice, rats and lemurs support these models, a different scenario may be more general among mammals in nature.

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CHAPTER 5

PRELIMINARY ANALYSIS OF A CONTACT ZONE BETWEEN KARYOTYPIC RACES OF THE COMMON SHREW (*SOREX ARANEUS*) IN SCOTLAND

S.J. Mercer and J.B. Searle

ABSTRACT. -- The Oxford and Aberdeen karyotypic races of the common shrew are characterised by the metacentric arm combinations *kq*, *no*, *pr* and *ko*, *np*, *qr* respectively. These races are parapatric in central Scotland, and to analyse the presumptive contact zone, we karyotyped 59 animals from 11 sites within 40km of the city of Stirling. To the east of the region sampled, we collected monomorphic Aberdeen and Oxford race samples within 8.5km of each other. To the west, two sites revealed Robertsonian polymorphism such that the Oxford race arm combinations were found in both metacentric and twin-acrocentric form. This suggests that a hybrid zone similar to that found between the Oxford and Hermitage races in southern England may occur in this region, whilst to the east, there may be a different type of contact zone, or a barrier between the two races.

INTRODUCTION

The complex karyotypic variation in the common shrew (*Sorex araneus*) has been the subject of much study. This variation has arisen as the result of a series of Robertsonian fusion mutations, each characterised by the fusion of a certain pair of single-armed (acrocentric) chromosomes to form a double-armed (metacentric) chromosome. Different acrocentric chromosomes have fused in different parts of the range of the common shrew, with the result that different metacentrics characterise different populations, and the species is divided into a number of 'karyotypic races' on this basis. Three of these are recognised in Britain, each named after the type locality (Searle 1984b). The Aberdeen race is found in

northern Scotland, Anglesey, southwest Wales, southwest England and the Isle of Wight (Searle 1988). It is distinguished by the presence of the metacentrics *ko* and *np* (nomenclature of Fredga and Nawrin 1977). The Oxford race occupies the south and east of Britain, and is characterised by the metacentrics *kq* and *no*. The Hermitage race occurs in a region intermediate between the other two races in the south of Britain, and possesses the metacentric *ko*. Each of these basic racial types is found with regional variations, the metacentrics *pr* and *qr* also present in some areas, as well as the acrocentrics *k*, *n*, *o*, *p*, *q*, and *r*. In addition, the metacentric *jl* is found to exhibit a low level of polymorphism, with a slightly higher frequency of the twin-acrocentric state in the south.

At the interfaces between races, a complex of chromosomal clines marks the change from one karyotype to the other. In some cases, there may be the presence of acrocentric chromosomes within the zone not found in either pure racial type, as with the hybrid zone between the Oxford and Hermitage races (Searle 1986). The presence of such acrocentrics reduces the frequency of 'complex' heterozygotes which are expected to form long chains or rings at prophase I of meiosis and to suffer from a degree of infertility (chapter 4).

Although the Oxford - Hermitage hybrid zone has been thoroughly studied and there is some information on the Hermitage - Aberdeen hybrid zone (Searle 1988; JB Searle and AJ Reilly, unpublished data), nothing is known of the zone between the Oxford and Aberdeen races, which is expected in central Scotland (Searle 1988). Each race possesses three

fusions not found in the other, the Oxford race with *kq*, *no*, and *pr* in this area, whilst the Aberdeen race has *ko*, *np*, and *qr*. Hybrids between these races have been generated in captivity (Searle 1984a), and are expected to form a ring VI configuration at prophase I of meiosis. The study of the Oxford - Aberdeen contact zone provides an interesting contrast to the Oxford - Hermitage hybrid zone.

MATERIAL AND METHODS

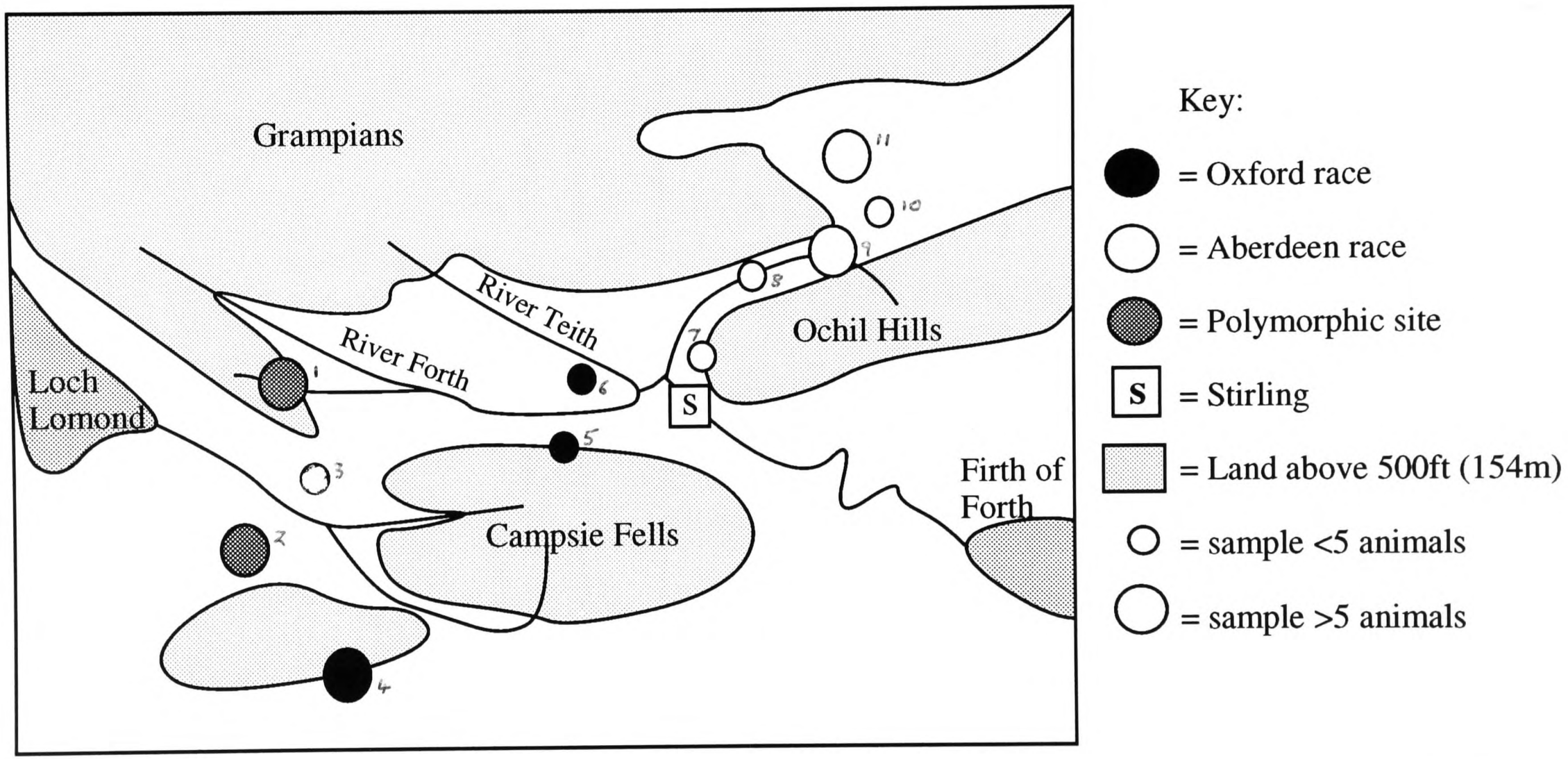
Animals were collected in unbaited Longworth traps (Chitty and Kempson 1949), checked at frequent intervals. All captures were fed and housed in the manner of Searle (1983) overnight, and sacrificed the following day. Mitotic preparations were made from bone marrow by the air-drying method of Ford (1966). Slides were aged and G-banded using the technique of Searle (1986), and microscopically examined at 1000X magnification under oil immersion. A minimum of five complete banded spreads were scored for each individual before the karyotype was recorded.

RESULTS

A total of 59 animals were collected from eleven sites in central Scotland (figure 1), and the frequency of each metacentric chromosome at all sites is presented in table 1. The majority of sites were found to be of one or other pure racial type, with two main areas of interest outlined below.

Polymorphism was only found in two sites, both to the southeast of Loch Lomond (figure 1): Dalmary and Drymen. All the Oxford race arm combinations (*kq*, *no*, *pr*) were present in

Figure 1. The contact zone between the Oxford and Aberdeen races in central Scotland



(Numbers adjacent to sample sites correspond with those in table 1, overleaf.)

Table 1. Trapsites in central Scotland

	Site	Grid reference[1]	No. Animals	Frequency of metacentrics							Race
				jl	*ko*	*np*	*qr*	*kq*	*no*	*pr*	
1	Drymen	NS 468 853	5	1	0	0	0	0.9	1	0.9	Oxford
2	Dalmary	NS 510 946	7	1	0	0	0	0.86	0.79	0.86	Oxford
3	Killearn	NS 518 885	1	1	0	0	0	1	1	1	Oxford
4	Blairskaith	NS 586 757	5	1	0	0	0	1	1	1	Oxford
5	Kippen	NS 650 920	3	1	0	0	0	1	1	1	Oxford
6	Doune	NS 730 978	4	1	0	0	0	1	1	1	Oxford
7	Stirling	NS 812 961	4	1	1	1	1	0	0	0	Aberdeen
8	Greenloaning	NN 818 058	4	1	1	1	1	0	0	0	Aberdeen
9	Blackford	NN 885 085	7	1	1	1	1	0	0	0	Aberdeen
10	Gleneagles	NN 904 113	4	1	1	1	1	0	0	0	Aberdeen
11	Muthill[2]	NN 910 180	15	1	1	1	1	0	0	0	Aberdeen

[1] References from the Ordnance survey Landranger series maps, 1:50,000 scale

[2] J.B. Searle and J.M. Wòjcik, unpublished data

both a metacentric and twin - acrocentric state in Dalmary, and one individual was a triple heterozygote. It is probable that Dalmary and Drymen are located at the southern edge of an area of polymorphism surrounding the putative Oxford - Aberdeen hybrid zone, akin to that found in association with the Oxford - Hermitage hybrid zone in southern Britain. Further east, in the immediate vicinity of Stirling (figure 1), we have found both the Oxford and Aberdeen races. The distance between the nearest Aberdeen and Oxford race sites, of Stirling and Doune respectively, is approximately 8.5km. No polymorphism is found in this region, indicating that if there is a hybrid zone here, it is extremely narrow. It is possible that the inter-racial boundary coincides with a river such as the Teith or the Forth, to the northwest and southwest of Stirling respectively, but neither is sufficiently large to serve as a complete barrier to dispersal. Nonetheless, it seems that the degree of introgression of either set of race-specific metacentrics is negligible in this region.

DISCUSSION

From our findings here, taken together with previous data for Scotland, (Searle 1984b; Ford and Graham 1964; Ford and Hamerton 1970; JB Searle and JM Wòjcik, unpublished data) we can now tentatively sketch the course of the Oxford - Aberdeen contact zone. The zone appears to follow low ground in the east from the region of Stirling northwestwards, probably along the valley of the river Teith. The zone then crosses the southern part of the Grampian mountains until it reaches the coast at some point between the sites of Dunstaffnage near

Oban (on the mainland, opposite the island of Mull), and Balmacara, near Kyle of Lochalsh (opposite Skye). A number of the more southerly of the Scottish islands have been sampled (Ford and Graham 1964; JB Searle and JM Wòjcik, unpublished data), and are assigned to the Oxford race. It is interesting to note that a polymorphism for the metacentric *pr* is found on the island of Islay.

The polymorphism observed at the sites of Drymen and Dalmary suggests a hybrid zone structure similar to that of the Oxford - Hermitage hybrid zone, at least for part of the region of contact between the Oxford and Aberdeen races. All the variable chromosome arms *k*, *n*, *o*, *p*, *q*, and *r* are found in an acrocentric state at Dalmary, despite the apparent lack of polymorphism within the main distribution of either bordering race (Searle 1984b). This suggests an 'acrocentric peak' similar to that involving the acrocentrics *k* and *o* in the zone between the Oxford and Hermitage races (Searle 1986). Presumably in the putative Oxford - Aberdeen hybrid zone, individuals with a substantially acrocentric karyotype are favoured over those with a hybrid karyotype involving several metacentrics with monobrachial homology, with the latter expected to suffer reduced fitness due to meiotic irregularities (see chapters 3 and 4).

We have documented in more detail the contact between the Oxford and the Aberdeen races to the east, in the region of Stirling. Here, there is no apparent polymorphism, and although sample sizes are small, our data indicate that there may be less than 8.5km between sites of the two monomorphic races. This contrasts with an area of polymorphism

approximately 100km in width for the Oxford - Hermitage hybrid zone. The region between the sites of Doune (Oxford race) and Stirling (Aberdeen race) is level fertile agricultural land, with no obvious barriers to dispersal, except for some small rivers. It may be, however, that the contact between races in this region is relatively recent, occurring only in historic times with the drainage of the lowland mires, an unfavourable habitat for the common shrew (Yalden 1982). In this case, it may be that introgression of metacentrics and the formation of a hybrid zone is just beginning, and more work is required to determine this.

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CHAPTER 6

CHARACTERISTICS OF CHROMOSOMAL CLINES OF THE COMMON SHREW (*SOREX ARANEUS*) IN SOUTHERN BRITAIN

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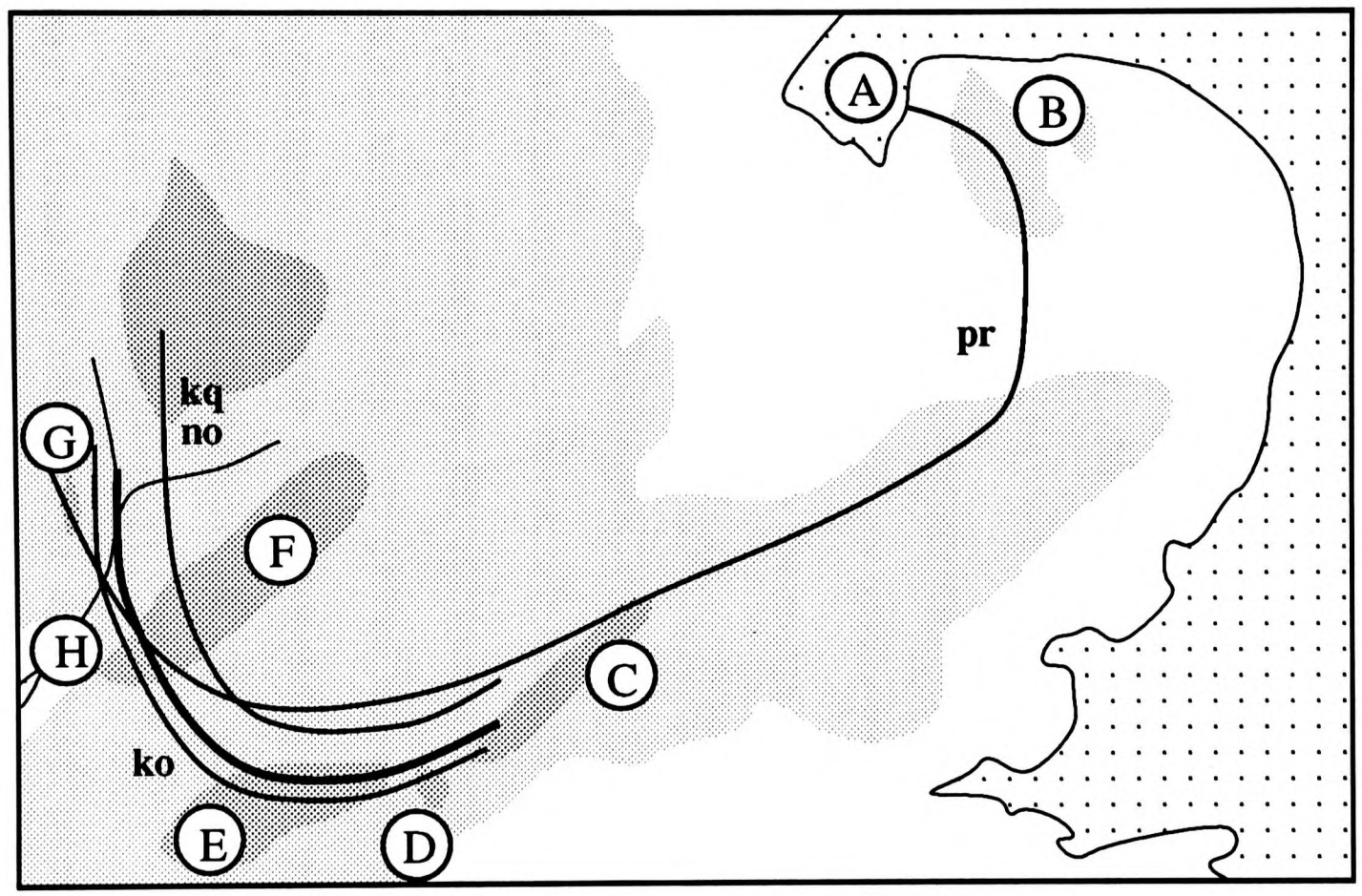
ABSTRACT. -- A total of 294 common shrews were collected from 49 sites in southern Britain, from Norfolk (in the east) to the Severn valley (in the west -- see figure 1). From these data, we were able to reconstruct the routes followed by four chromosomal clines in the region. The clines for *ko*, *kq* and *no* are found as components of the elaborate hybrid zone between the 'Oxford' and 'Hermitage' chromosome races, and run southwards from the vicinity of Birmingham, following the Severn valley before crossing the Cotswold Hills and associating with the northernmost ridge of the Berkshire Downs eastwards to the Goring Gap (figure 1). The cline for *pr*, whilst associated with the hybrid zone in the region of the Berkshire Downs, diverges to the west, associating with the Malvern Hills, and to the east, where it follows the curving chalk ridge running through East Anglia northeastwards to the Wash. In all cases, the clines exhibit characteristics consistent with maintenance through an environmentally independent heterozygote disadvantage.

Widths were calculated for all clines between the 12 and 88% isoclines (8.6km (*ko*), 28.4km (*kq*), 37.1km (*no*), 47.9km (*pr*)). No difference was noted between the width of the cline of chromosome *pr* when in association with others (as in the Oxford-Hermitage hybrid zone) as opposed to alone. The degree of heterozygote disadvantage calculated from cline width accords well with values established from previous meiotic studies.

Figure 1. Cline paths in southern Britain



50 miles
(80.5 km)

Key:

- Land over 400 ft (122 metres)
- Land over 200 ft (61 metres)
- Cline centres (50% isoclines)
- Hybrid zone centre
- (A) The Wash
- (B) East Anglian Heights
- (C) Chiltern Hills
- (D) Goring Gap
- (E) Berkshire Downs
- (F) Cotswold Hills
- (G) Malvern Hills
- (H) River Severn

INTRODUCTION

The existence of chromosomal variation in populations of the common shrew has been known for many years (Ford et al. 1957). The ancestral karyotype had an extreme tendency to undergo Robertsonian fusion (Volobouev 1989), such that different assemblages of metacentric chromosomes exist in different areas (Searle 1984a, 1988a). Such distinct chromosomal assemblages are called chromosomal 'races' (Searle et al. 1990).

Of the three such races found in Britain, we are here concerned with the Oxford race (occupying central and northern England), and the Hermitage race (found in a band from north Wales to southeastern England, Searle 1984a, 1988a). These two races are distinguished by different Robertsonian fusions involving some of the smallest chromosome arms in the karyotype, such that the Oxford race possesses the metacentrics *kq* and *no*, whilst the Hermitage race has the metacentric *ko* and acrocentrics *n* and *q* (nomenclature of Searle et al. 1991). The chromosome arms *p* and *r* occur combined as a metacentric over much of the range of both the Oxford and Hermitage races, but as acrocentrics in some areas, most notably East Anglia. Here, it gives rise to a regional variant of the Oxford race and the formation of a cline of metacentric frequency, from absence in the east to fixation in the west (see figure 2 in chapter 1).

Chromosomal clines of this type are most probably maintained through a mechanism of selective disadvantage, acting on individuals possessing chromosomal homologues in both metacentric and acrocentric form. Studies have been made

of the effects of Robertsonian heterozygosity on fertility in both the male and the female common shrew. In the male, data from testis weights and primary spermatocyte to round spermatid ratio support the contention that germ cell death increases with the degree of chromosomal heterozygosity (Searle 1988b; Garagna et al. 1989; chapter 4). Aneuploidy in the male (Searle 1986a, 1988b; chapter 3) shows no elevation in simple heterozygotes, but the limited data available for heterozygotes with monobrachial homology (chapter 4) indicate that aneuploidy is increased in these more complex hybrid animals. In the female (Searle 1990), the range of nondisjunction determined from fetal losses for homozygous animals is lower than that for simple heterozygotes. In summary, although there is evidence to suggest that heterozygosity for Robertsonian fusions causes disruption of meiosis in the common shrew, this effect is slight until the multivalent configurations become large. When we consider the widths of chromosome clines in the common shrew, however, it is evident that the degree of disadvantage required for their maintenance may indeed be small.

The *pr* cline in the common shrew represents the simplest form of cline. Populations at the centre of such a cline will possess the highest proportion of Robertsonian heterozygotes, and therefore incur a greater disadvantage than their neighbours. Based on cline theory (Endler 1977; Barton 1979), one would expect the centre of this cline to become localised where population density is low (see chapter 1). Furthermore, the width of the cline will be determined by a balance between

dispersal and the degree of heterozygous disadvantage (Barton 1979).

The term 'hybrid zone' is here used to describe a situation in which a number of clines in close proximity form the interface between two chromosome races of common shrew. Such a zone is therefore influenced not only by those factors governing the behaviour of a single cline, but also by interactions between clines. For example, there will be a tendency for the relatively disadvantaged populations at the cline centres to attract each other, particularly clines of similar gradient, which will tend to become associated with the same geographical features.

In the Oxford - Hermitage hybrid zone, the clines for the Oxford race metacentrics *kq* and *no* would be expected to overlap that of the metacentric *ko*. Because of the monobrachial homology between *ko* and *kq* or *ko* and *no*, this overlap would be expected at 50% frequency of all the metacentrics; in essence, the clines would be locked together. Therefore, the populations at the centre of this zone would be expected to have the highest proportion of individuals heterozygous for (in this case) *ko*, *kq* and *no*. Given the presence of the acrocentrics *n* and *q* of the Hermitage race, such 'complex' heterozygotes should form a chain of five chromosomes during prophase I of meiosis. 'Complex' heterozygotes are expected to suffer a more severe fertility penalty than 'simple' heterozygotes (see chapters 3 and 4), therefore the populations at the core of the hybrid zone will be at a greater disadvantage than those in the clines immediately bordering it, which would in their turn be

disadvantaged in comparison to the homozygotes in the surrounding populations.

In the case of the Oxford-Hermitage hybrid zone, however, there is a further level of complexity. At the centre of the zone we find populations of shrews possessing the acrocentric chromosomes *k* and *o*, not found in either bordering race. The origin of these acrocentrics, whether a result of fission of a Robertsonian metacentric, or predating one or both of the fusions in the bordering races, is obscure. It would seem that their retention is due to the role they play in the reduction of the frequency of complex heterozygotes, and their frequency is highest where the highest proportions of all three monobrachial homologues are found (Searle 1986b). As a consequence, the clines for *ko*, *kq* and *no* are not linked together at their 50% isoclines, overlapping instead at about 20% (Searle 1986b; see figure 5b in chapter 1).

The nature and behaviour of chromosomal clines has been studied in theory, but practical demonstrations are rare (see references in Shaw 1981 for insects; Searle, in press for mammals). In the common shrew, however, these situations are relatively common, and here we present both the simple case of one cline, and the more complex case of a hybrid zone, to examine the adherence of the natural situation to the theory.

MATERIAL AND METHODS

Common shrews were collected from localities throughout the study area from autumn of 1988 to autumn 1990. Collection was by means of unbaited Longworth traps (Chitty and Kempson 1949), checked hourly. Common shrews caught in this manner

were maintained for up to two days on a diet of minced Ox heart prior to being sacrificed by cervical dislocation. Bone marrow from both femurs was used for karyotyping by the method of Searle (1986a), and the air-dried slides produced were aged before being G-banded (method of Searle 1984b). A minimum of five such banded mitotic spreads were examined under the light microscope at 1000X magnification (and oil immersion), and the karyotype recorded.

Sites sampled were scattered across the study area, and were first examined to give an estimate of the path of each cline by eye. From this, it was possible to construct a number of transects running at right angles to each cline. A convention was adopted in each case to include sample sites up to 10km to either side of the midline of the transect. A hyperbolic tangent curve was fitted to each transect using a proprietary curve-fitting computer program, allowing accurate measurements to be made of the cline width (taken as the distance between the 12 and 88% frequency isoclines: Barton and Hewitt 1981), and the goodness of fit of the curve, expressed as a Chi-squared value (see table 3). Cline widths were averaged (in cases where more than one estimate was possible from the data), and the resultant width estimate used to more accurately plot the path of the 50% isoclines (figures 2 and 3).

RESULTS

The frequency of presence of each variable Robertsonian metacentric was calculated for each trapsite. These values are given in tables 1a, 1b and 2.

Table 1a. Frequencies of the metacentric chromosome *pr* at sites in the East Anglian area

Site	Grid reference[1]	Number of animals	Frequency of *pr*
Hatfield Forest[2]	TL 540,200	3	0.17
Needingworth	TL 364,740	5	1.00
Coveney	TL 484,800	3	1.00
Feltwell	TL 672,925	4	1.00
Lakenheath	TL 688,812	4	1.00
Barrow	TL 755,647	10	0.25
Euston	TL 913,802	5	0.30
Hilborough	TL 784,984	8	0.63
Shelfanger	TM 114,835	7	0.00
Mellis	TM 108,738	2	0.00
Badwell Ash	TL 999,694	4	0.38
Prickwillow	TL 613,837	4	0.75
Chatteris	TL 386,884	7	1.00
Marham	TF 735,105	8	0.56
Bittering	TF 941,167	10	0.40
Cockley Cley	TF 782,012	6	0.92
Roydon	TF 686,235	5	0.80
Tottenhill Row	TF 635,122	5	1.00
West Raynham	TF 853,258	3	0.50
Terrington St Clement	TF 535,235	5	1.00
Shernbourn	TF 705,343	5	0.60

[1] References from the Ordnance Survey Landranger series maps, 1:50,000 scale.

[2] The metacentric *kq* was found with a frequency of 0.67 at this site.

Table 1b. Additional sites used for the calculation of widths of the cline *pr* (JB Searle, unpublished data)

Site	Grid reference[1]	Number of animals	Frequency of *pr*
Twyford	TG 007,240	6	0.50
Southrepps	TG 266,363	4	0.00
UEA (Norwich)	TG 190,073	8	0.13
Stanhoe	TF 800,370	9	0.50

[1] References from the Ordnance Survey Landranger series maps, 1:50,000 scale.

East Anglia

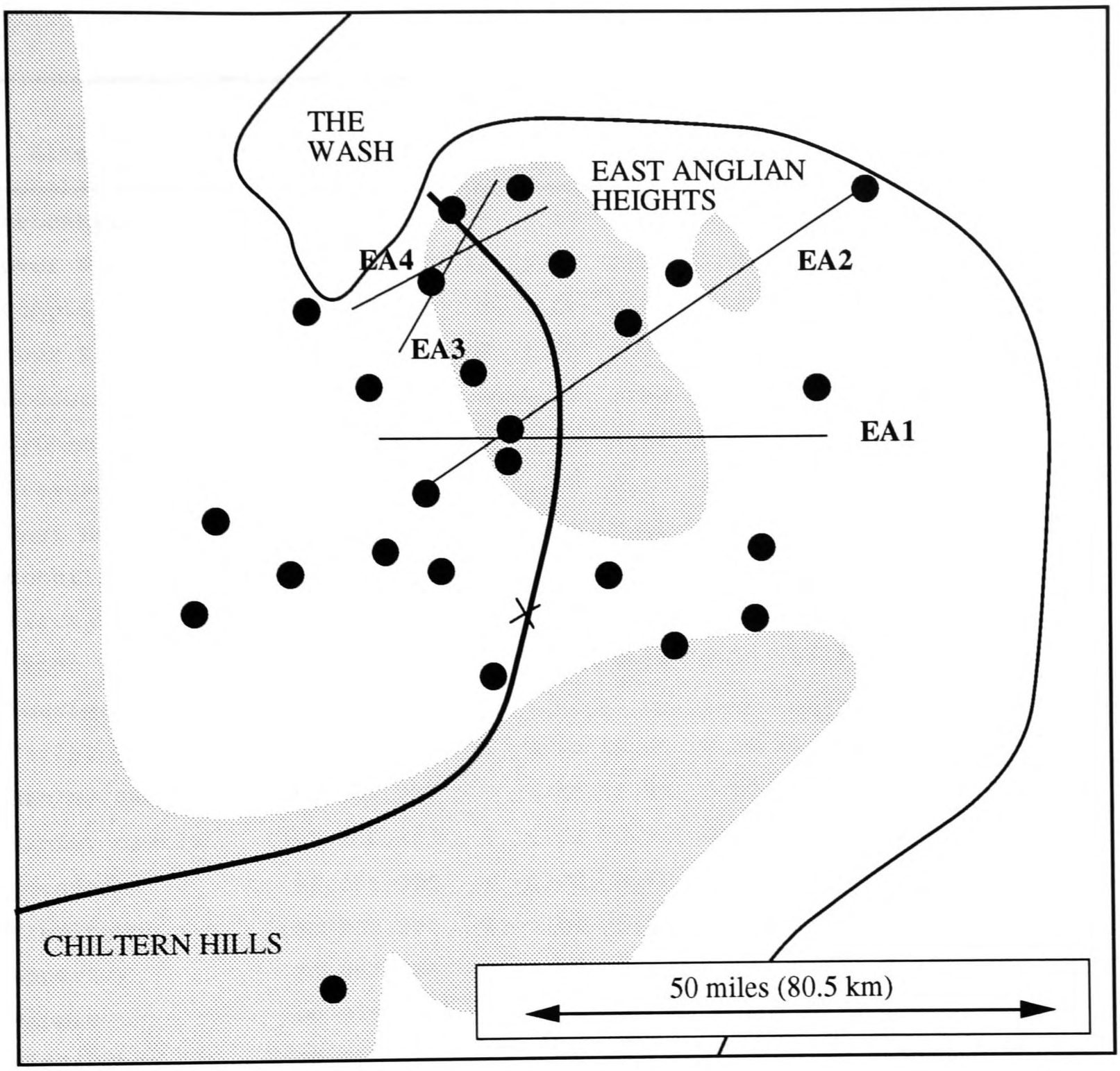
Transects constructed along the cline of the metacentric *pr* in northern East Anglia (tables 1a and 1b; figure 2), in addition to one from the Oxford-Hermitage hybrid zone (figure 3), give an average cline width (between the 12 and 88% isoclines) of 47.9km (table 3).

The 50% isocline appears to make contact with the coast on the eastern side of the Wash and to run southwestwards along the relatively high land of the East Anglian heights (figure 2). Where this land falls to the south, the zone crosses the Breckland in a virtually straight line, although it may localise at one point on the high land of Frog Hill (map reference TL 87,91: all references from Ordnance Survey Landranger series maps, 1:50,000 scale). On leaving the Breckland to the south, the zone begins to curve progressively more sharply to the west, following the western slope of the chalk ridge that leads into the Chiltern Hills.

Oxford-Hermitage hybrid zone

The scatter of trapsites allows the construction of a north-south transect across the hybrid zone to the south of Oxford (figure 3), and allows estimates to be made of cline widths for all the metacentrics involved (table 3). From these values, further sample sites (table 2), and some additional data (JB Searle and Y Luo, unpublished observations), it was possible to trace the path of the clines comprising the Oxford-Hermitage hybrid zone from the Goring Gap (west of the Chilterns) to the Malvern Hills.

Figure 2. The cline for the metacentric *pr* in East Anglia



Key:

Centre (50% isocline) of the *pr* cline

Land above 200ft (61 metres)

Midline of transect

Sample site

Frog Hill (TL 870, 910)

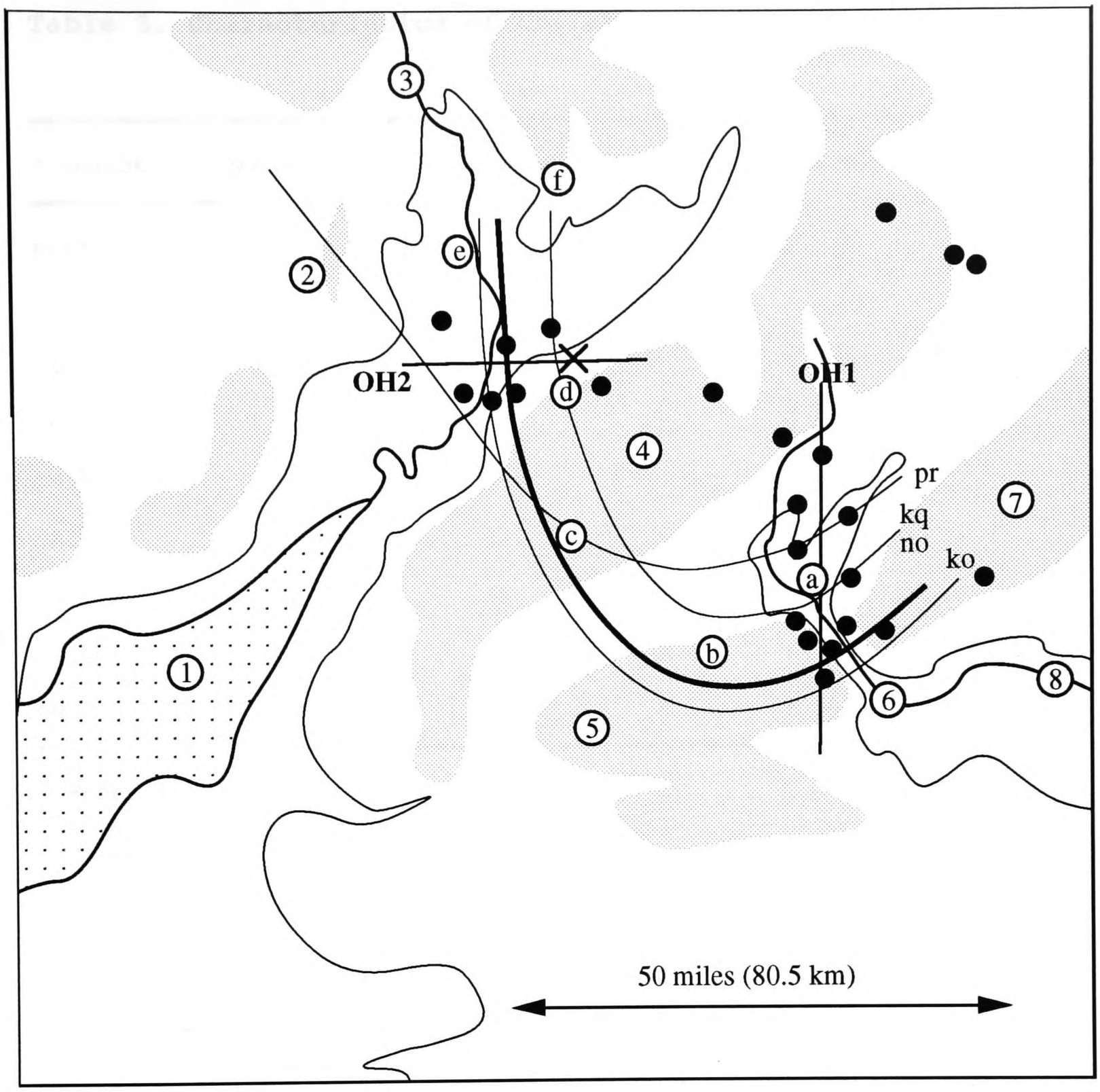
Table 2. Frequency of metacentric chromosomes at sites sampled in the area of the Oxford-Hermitage hybrid zone

Site	Grid reference[1]	N[2]	Frequency of metacentrics				
			jl	*kq*	*no*	*pr*	*ko*
Brockhall	SP 630,620	25	1.00	0.96	1.00	0.96	0.00
Over Norton	SP 310,290	11	0.95	0.68	0.77	0.91	0.00
Compton	SU 524,803	4	1.00	0.13	0.50	0.00	0.00
Four Elms	SU 513,748	9	1.00	0.00	0.00	0.06	0.83
Hodcott Down	SU 483,822	5	0.90	0.00	0.30	0.00	0.30
Wytham Wood	SP 458,081	3	1.00	1.00	0.83	0.67	0.00
Brasenose Wood	SP 557,056	8	1.00	0.63	0.88	0.75	0.00
East Hendred Down	SU 458,852	9	1.00	0.06	0.17	0.39	0.06
Cothill	SU 460,990	6	1.00	0.50	0.50	0.33	0.00
Salcey Forest	SP 790,523	3	1.00	1.00	1.00	1.00	0.00
Aston Upthorpe	SU 551,847	3	1.00	0.17	0.17	0.33	0.17
Courteenhall	SP 759,538	1	1.00	1.00	1.00	1.00	0.00
Tirley	SO 841,287	5	1.00	0.00	0.00	1.00	0.60
Hanley Swan	SO 802,426	7	1.00	0.00	0.00	0.50	0.86
Elmley Castle	SP 000,413	7	1.00	0.21	0.14	0.93	0.00
Cutsdean	SP 095,305	8	1.00	0.56	0.56	0.94	0.00
Tredington	SO 930,300	3	1.00	0.50	0.50	1.00	0.00
Apperley	SO 857,285	3	1.00	0.00	0.00	0.67	0.33
Ripple	SO 875,373	1	1.00	0.00	0.00	1.00	0.50
Piddington	SU 810,940	5	1.00	0.00	0.10	0.00	0.00
Ipsden	SU 620,840	6	1.00	0.33	0.08	0.08	0.00
Bletchingdon	SP 510,170	4	1.00	1.00	1.00	0.50	0.00
Wootton	SP 437,207	8	1.00	0.56	0.94	0.81	0.00
Little Wittenham	SU 561,934	10	1.00	0.35	0.40	0.50	0.00

[1] References from the Ordnance Survey Landranger series maps, 1:50,000 scale.

[2] Number of animals karyotyped

Figure 3. Clines in the Oxford-Hermitage hybrid zone



Key:

×	Bredon Hill	a	Oxford		Land above 400ft (122 metres)
1	Severn Estuary	b	Swindon	——	The 200ft (61 metre) contour
2	Malvern Hills	c	Cirencester	**OH1**	Transect
3	River Severn	d	Winchcombe	●	Sample site
4	Cotswolds	e	Worcester	pr	Cline of metacentric frequency
5	Berkshire Downs	f	Reddich	▬▬	Centre of the hybrid zone
6	Goring Gap				
7	Chiltern Hills				
8	River Thames				

Table 3. Characteristics of chromosomal clines

Transect	Chromosome	No. Sites in transect	Width (km)	Goodness of fit[c]
EA1[a]	*pr*	5	43.9	0.0106
EA2[a]	*pr*	8	55.1	0.0249
EA3[a]	*pr*	5	43.7	0.0036
EA4[a]	*pr*	6	41.1	0.0044
OH1[b]	*pr*	11	55.6	0.0271
OH1[b]	*kq*	11	28.4	0.0263
OH1[b]	*no*	11	37.1	0.0199
OH1[b]	*ko*	11	9.0	0.0119
OH2[b]	*ko*	6	8.1	0.0011

[a] Refer to figure 2 for location of transect.
[b] Refer to figure 3 for location of transect.
[c] expressed as a Chi-squared value.

Average cline characteristics

Chromosome	Width (km)[a]	s[b]
kq	28.4 (26.0)	0.000099
no	37.1 (31.0)	0.000058
pr	47.9 (53.0)	0.000035
ko	8.6 (17.0)	0.001100

[a] Figures in brackets from Searle (1986b)
[b] Where **s** is the fitness deficit in simple heterozygotes (Barton and Hewitt 1981), assuming the dispersal distance per generation to be 100 metres (JB Searle and AJ Reilly, unpublished observations).

The 50% isoclines for the Oxford race-specific metacentrics *kq* and *no* (cline widths 28.4 and 37.1km respectively -- table 3) occur together, both lying on a ridge which rises to 100 metres, about 8km south of the city of Oxford, but still about 15km to the north of the first high ridge of the Berkshire Downs. These clines remain coincident, and run parallel to the northern ridge of the Berkshire Downs westward to the area of Swindon (SU 30,80), where they cross the upper Thames valley and intersect the central ridge of the Cotswolds at right angles, leaving these hills in the vicinity of Winchcombe (SP 03,30). To the north of the Cotswolds, they become parallel with the river Severn, but their 50% isoclines pass approximately 15km to the east of Worcester, possibly associating with a ridge extending due south from Reddich (SP 06,67).

The *ko* cline appears to run parallel with those of *kq* and *no* in the vicinity of Oxford, but with a centre approximately 23 km to the south (and south of the ridge of the Berkshire Downs). In the vicinity of the Severn valley, the cline centre again parallels those of *kq* and *no*, appearing to be localised on the river itself. It should be noted that although only small numbers of animals were obtained from the sites closest to the east bank of the Severn, they clearly show that both *ko* and the acrocentrics *k* and *o* have crossed the river (sites of Apperley and Ripple; table 2).

The location of the 50% isocline of *pr* is less clear from these data, but appears to be 19km north of the centres of the *kq* and *no* clines in the vicinity of Oxford, and not clearly associated with any geographical feature. It continues to

parallel the course of these two clines to the point where they curve northwards, but there the *pr* cline continues westwards along the lowlands of the upper Thames valley to the vicinity of Cirencester (SP 03,02), where it turns sharply to the north, cutting across the Cotswolds, and possibly associating with the river Severn in the south, before veering westwards again to become localised on the Malvern Hills.

DISCUSSION

Factors influencing the path of clines

In East Anglia, it seems that the path of the cline for the metacentric *pr* is dictated by the crescent-shaped ridge of chalk that forms the relatively high land to the east and north (figures 1 and 2). This course is consistent with the influence of different habitats on a zone maintained through heterozygote disadvantage. In the north of the region, the zone shows localisation on a distinct geographical feature, as the 50% isocline clearly follows the central ridge of the East Anglian Heights, which rise to 87 metres (figure 2). As this land falls away to the south and the cline enters the Breckland, it exhibits the properties of a tension zone (Key 1968), travelling in straight lines to minimise the area of contact with either bordering population. To the south of this, as the chalk ridge broadens, the cline centre is associated with the northern and western slopes, closely parallel to the 50 metre contour, its course in this area dictated by the boundary between the hilly habitat of the south and east, and the lowlands to the north and west (figure 1).

The clines in the Oxford-Hermitage hybrid zone (*ko*, *kq* and *no*) may be expected to act as a unit, bound together by a mutual attraction (and an affinity for similar geographical features), but separated by the presence of a peak of acrocentric chromosomes in the centre of the zone. It may therefore be expected that although each individual cline centre may be drawn towards local geographical features, the greatest influence on the path of the zone will be populations in which the proportions of all three metacentric chromosomes sharing an arm in common (and hence producing the greatest number of complex heterozygotes) is highest.

Directly to the south of Oxford, the population possessing the highest proportions of *ko*, *kq* and *no* occurs in the region of the northernmost ridge of the Berkshire Downs (figure 3). This point marks the boundary between the extensive flat land of the upper Thames valley and the successive ridges of high land that extend almost to the south coast (figure 1). The hybrid zone may therefore be said to run parallel to the change in habitat type, and not the ridge of the Berkshire Downs alone. As has previously been explained, the clines for *ko*, *kq* and *no* form a unit, and the localisation of the hybrid zone leaves the cline centre of *ko* to the south, and those of *kq* and *no* to the north, and in neither case is association with a geographical feature clear. The cline of *pr* passes to the north of this complex, associated in the East with the Chiltern Hills, and the Cotswolds to the west (figures 1 and 3).

Sampling in the Severn valley reveals a similar picture. The obvious feature for the localisation of a zone is the

barrier to dispersal afforded by the river Severn itself, and chromosome frequencies indicate that this is indeed the case. Consequently, the cline centres of *kq* and *no* are found to the east, and the centre of *ko* to the west. In the former case, it seems that the cline centres may, in addition to being governed by the position of the hybrid zone, also have become localised on a range of isolated but steep hills (the largest being Bredon Hill, rising to 293 metres) to the south (figure 3).

Comparison of cline widths

Measurements of cline widths from the Oxford area have previously been made by Searle (1986b), and are in general agreement with the data from the present study (table 3). With regard to the main discrepancy, the width of the *ko* cline, the value of Searle is supported by a greater number of samples containing *ko*.

The width of a cline, and hence its gradient, is the product of the dynamic equilibrium between the spreading effect of dispersal of animals from the zone and the degree of unfitness of heterozygous carriers (Barton 1979). Whilst the heterozygote unfitness may be assumed to remain constant, dispersal may vary with habitat. Comparison between the width values obtained for the *pr* cline in East Anglia and in the Oxford area show good agreement (table 3) and indicates that differences in dispersal affect the width of a cline only to a minor extent (at least when those differences are small, as they presumably are between the farmland of East Anglia and that of Oxfordshire). A second conclusion to be drawn from the

close agreement of the East Anglian and the Oxford cline widths is that whereas the East Anglian *pr* cline is solitary, in the Oxford area it forms a part of the complex of clines making up the Oxford-Hermitage hybrid zone. Partially overlapping the *kq* and *no* clines has not detectably reduced the width of *pr*, as might be expected if heterozygotes for several fusions were affected much more severely than heterozygotes for one.

Our data support the view that the gradient of each cline of Robertsonian chromosome frequency reflects the degree of heterozygote disadvantage imposed by that fusion. Given the cline widths, and taking 100 metres as a likely value for dispersal per generation (JB Searle and AJ Reilly, unpublished observation), the equation of Barton and Hewitt (1981) gives an estimate of the degree of fertility depression required to maintain such clines (table 3). From these calculations, the degree of fertility depression in all types of heterozygote is expected to be extremely small. This is in keeping with the results reported in chapter 3 for males, and Searle (1990) for females, which showed only slight differences in fertility between homozygotes and heterozygotes.

The frequency of the most complex heterozygotes found in the hybrid zone (with *kq*, *no* and *ko*, and hence forming chains of five at prophase I of meiosis) is reduced by the presence of the 'acrocentric peak' of *k* and *o* in the centre of the zone, and the frequency of these acrocentrics can rise to 80% (Searle 1986b). As a consequence of this, the rarity of chain-forming animals calls into question whether the populations in which chains may be expected to occur will be as severely

affected as those with a much higher frequency of more mildly affected heterozygotes. Using the Hardy-Weinberg equilibrium, it is possible to calculate that only 3.3% of animals can be expected to form chains in the most severely affected populations. An estimate of the disadvantage incurred from such a chain can be obtained from studies of captive-bred chain VII heterozygotes (chapter 4), where the elevation of nondisjunction above background is in the region of 10%. A similar value for the *kq-ko-no* chain of five would give a decrease in fertility of the population as a whole of 0.0033% due to the chains alone (a minimum value, not taking into account simple heterozygotes or those complex heterozygotes forming smaller chains). This compares with a decrease in fertility of 0.00055% for the most severely affected simple heterozygote population (at the centre of the 50% isocline of *ko*, a maximum value, calculated using the narrower of the two estimates of cline width, see table 3), and hence supports the conclusion that the most disadvantaged populations lie at the centre of the hybrid zone (and litter size is smaller in populations at the centre of the hybrid zone in comparison to flanking populations -- Searle 1990). Although these conclusions are consistent with our current knowledge, more data are required to establish with precision the course of each chromosome cline (such as that achieved with *Podisma* -- Barton and Hewitt 1981), the population densities of shrews in different habitat types, and the validity of heterozygote disadvantage as the principal factor in cline maintenance.

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CHAPTER 7

CONCLUSIONS AND GENERAL DISCUSSION

NATURE OF THE POLYMORPHISM

It is commonly thought that karyotypic heterozygotes are at a disadvantage relative to homozygotes. In the common shrew, this study has shown that the fertility disadvantage in simple Robertsonian heterozygotes is (at most) extremely small (chapter 3). Cline widths in the common shrew are all extremely large in relation to dispersal distance, and analysis has shown that the degree of disadvantage in the case of simple heterozygotes is sufficient to maintain the clines studied here (chapter 6). However, the set of metacentric chromosomes comprising the Oxford race are separated from the Hermitage race-specific chromosomes in the Oxford-Hermitage hybrid zone. This separation is brought about by means of a high frequency of acrocentric elements which are confined to the zone and thought to be maintained as a result of selection against complex heterozygotes (Searle 1986). In this study, those complex heterozygotes generated did suffer higher germ cell death and anaphase I nondisjunction than reported for simple heterozygotes (chapter 4).

Geographical mapping has shown that the cline associated with polymorphism for the metacentric *pr* behaves as theory dictates, associated with local areas of expected low shrew density and suspected discontinuities in habitat. Similar behaviour has been noted for the more complex assemblage of clines forming the Oxford-Hermitage hybrid zone (chapter 6). Of all the clines studied, that for the metacentric *pr* had the greatest width, and in this case approaches the value given for a wave of advance of a neutral polymorphism by Barton and Bengtsson (1986). It is interesting to observe that although

pr agrees with theories of cline behaviour in those areas studied, it exhibits the only known case in Britain of introgression of one metacentric into another race, crossing the hybrid zone in North Wales and achieving fixation in the northwestern populations of the Hermitage race (chapter 1 figure 2; Searle et al. 1990). It is possible that in this instance the heterozygote disadvantage is so mild that the resultant cline is only loosely constrained by the forces that govern cline behaviour.

PRIMARY OR SECONDARY CONTACT?

One of the critical questions about clines is whether they have a primary or secondary origin (Endler 1977). In the case of chromosome races of the common shrew in Britain, it is important to establish whether these clines arose (a) through secondary contact within Britain, (b) formed by primary differentiation within Britain, or (c) arose outside Britain and moved in. The common shrew would have spent the last glaciation in southern Europe and so possibilities (a) and (b) would have had to occurred within the last 15,000 years (see chapter 1 and references therein).

I have taken the view that the ancestral races are Oxford and Aberdeen (chapter 1), with the Hermitage race arising through modification of the hybrid zone (supported by findings of a direct Aberdeen-Oxford contact in Scotland -- chapter 5). Given the disjunct distribution of the Aberdeen race, with isolates in northern Scotland, Anglesey, southwest Wales, southwest England, and the Isle of Wight, this implies a wave of displacement of the Aberdeen race by either the Oxford or

the hybrid Hermitage race. With regard to models (a) and (b), there is no clear reason why there should be displacement of one race by another. For model (c), moving clines entering Britain - displacement would be just a continuation of expansion (Searle and Wilkinson 1987).

INCIPIENT SPECIATION?

The most likely heterozygotes to have reduced fertility are those complex heterozygous individuals which form rings or long chain configurations at prophase I of meiosis. As I have demonstrated, even these individuals may have relatively mild infertility in the common shrew (chapter 4). This factor, coupled with the reduced frequency of such individuals both in the Oxford-Hermitage hybrid zone and apparently in the Oxford-Aberdeen zone due to acrocentric peaks (chapters 5 and 6), make speciation an unlikely possibility. The average fertility of individuals in these hybrid zones is likely to be so high (see Searle 1990) that gene flow will hardly be impeded.

Although this is true for the hybrid zones in Britain, it need not be so for all hybrid zones in the common shrew. The Tomsk and Novosibirsk races of Soviet central Asia differ such that hybrid individuals would be expected to form chain configurations of up to nine elements at prophase I of meiosis (Aniskin and Lukianova 1990). The hybrid zone appears to be very narrow, with no acrocentric peak. Obviously it is of importance to measure the fertility of the hybrids in this case to determine whether it is similar to that found in chain VII-forming Oxford-Aberdeen hybrids (chapter 4). In any case, gene flow is likely to be more heavily impeded in this zone

than in the Oxford-Hermitage hybrid zone, and speciation consequently more probable.

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Multiple paternity in wild common shrews (*Sorex araneus*) is confirmed by DNA-fingerprinting

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We have tested for the occurrence of multiple paternity in wild common shrews by karyotypic analysis and DNA-fingerprinting of five wild-caught females and their litters. Karyotypic data suggest that some litters were sired by more than one male, but provide no definitive evidence. By using DNA-fingerprinting, it was possible to establish that two males sired the litter of two females. The present report shows that multiple paternity is not a rare phenomenon in the common shrew and by using DNA-fingerprinting it is possible to assign individual offspring to different male parents even when none of the putative fathers are available for inspection.

Keywords: DNA-fingerprinting, karyotypic analysis, multiple paternity, *Sorex araneus*.

Introduction

Common shrews (*Sorex araneus*) are extremely difficult to observe in their natural surroundings and there is therefore no substantial information on their mating behaviour in the wild. Ecological studies indicate that male common shrews do not normally have breeding territories but tend to be nomadic; presumably searching out or making themselves available to as many females as possible (Shillito, 1963; Michielsen, 1966). Male shrews do not contribute to the maintenance of the female or her young. Observations on captive, sexually-mature females (Dehnel, 1952; Crowcroft, 1957) indicate that they are sexually receptive for a period of 2 h and at other times are hostile to the male. In the laboratory, the observation that a male will mount a female a large number of times (Dehnel, 1952) suggests the opportunity for copulation with different males and the possibility for more than one male to sire offspring within one litter.

Multiple paternity has been demonstrated in the common shrew by karyotypic analysis of individuals caught in a region where Robertsonian chromosomal polymorphism is present (Searle, 1990). From the karyotypes of wild caught pregnant females and their offspring it could be shown that more than one male contributed to 4 out of 16 pregnancies analysed. Studies of a large number of female common shrews provide no evidence that embryos are retained from one pregnancy to the next (Brambell, 1935; Searle, 1984; Tarkowski, 1957) and thus, the karyotypic analysis indicates that different males must have sired offspring during the short period when females are receptive.

From the typing of mother and offspring with simple Mendelian markers, such as chromosomes and enzyme variants, multiple paternity is demonstrated when more than two 'alleles' are shown to be transmitted on the male side. However, due to a generally low level of detectable variability in chromosomes and enzymes, studies of such markers are likely to underestimate the level of multiple paternity. Using methods where the level of detectable genetic variation is higher would enable a more detailed assessment of paternity. Polymorphisms in tandem-repetitive DNA-sequences ('minisatellites'), resulting from allelic variation in the number of repeats, show an extremely high level of heterozygosity, often exceeding 60 per cent (Nakamura *et al.*, 1987). DNA probes isolated from a subset of human minisatellites with a core sequence of 10–15 bases (Jeffreys, 1987) and a cluster of 15-base repeats from the wild type M13 phage (Vassart *et al.*, 1987)

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establish complex, individual-specific fingerprints (Jeffreys *et al.*, 1985). These DNA-probes reveal high levels of genetic variability in various animals (Burke & Bruford, 1987; Jeffreys & Morton, 1987; Jeffreys *et al.*, 1987; Wetton *et al.*, 1987; Georges *et al.*, 1988; Weiss *et al.*, 1988; Burke *et al.*, 1989).

In this report we have applied genetic fingerprinting using two highly variable minisatellite DNA-probes and analysis of Robertsonian chromosomal variation, to assess multiple paternity in the common shrew.

Methods

Animals

Five pregnant females were collected live from grassland sites near Oxford, UK and subsequently maintained in isolation. Female 2087 was caught in East Hendred while the others were from Cothill (site details in Searle, 1986). Females were allowed to rear their young until weaning and then the female and complete litters were killed and karyotyped according to the method of Searle (1986). Animals were stored at −20°C until isolation of DNA. Altogether 36 individuals were fingerprinted (female no. 2087 + 7 young, 2097 + 10 young, 2098 + 6 young, 2102 + 4 young and 2103 + 4 young). One additional member of the litter of female 2103 was karyotyped but not fingerprinted. Female 2097 and her young were not subjected to chromosomal analysis.

DNA extraction

Five-hundred microlitres of SET-buffer (0.15 M NaCl, 0.05 M Tris, 1 mM EDTA, pH 8.0, autoclaved) were added to the samples (0.3–0.4 g of muscle tissue) and placed in a +55°C waterbath. Then, 25 μl of 25 per cent (w/v) SDS and 75 μl of Proteinase K (15 units sample^{-1}) were added. The tubes were shaken gently and left for 1–2 h at +55°C. DNA was purified by two extractions with phenol/chloroform and two with chloroform. DNA was precipitated with 99 per cent ethanol at −20°C and pelleted at 12,000 ***g***, washed with 70 per cent ethanol and vacuum dried. The DNA was dissolved overnight in 400 μl of TE-buffer (10 mM Tris, 1 mM EDTA, pH 7.0, autoclaved). DNA (8–10 μg in 400 μl) was digested with 30 units of Hae III for 5 h at 37°C, extracted once with phenol/chloroform, once with chloroform and then precipitated as above. The digested DNA was redissolved overnight in 25 μl of TE. Electrophoresis of DNA-fragments was performed in 0.8 per cent agarose gels for 24–28 h at 1.7 V cm^{-1} and transferred to Biodyne nylon membranes by vacuum blotting.

Hybridization

The insert of human minisatellite clone 33.15 (Jeffreys *et al.*, 1985) and a 2900 fragment from wild type M13 (Vassart *et al.*, 1987) were isolated by preparative restriction enzyme digestion and electrophoresis in low melting-point agarose. The probe DNA was purified from the agarose using Gene Clean (Bio 101). Probe DNA (50–100 ng) was labelled within ^{32}P by the random primer method (Feinberg & Vogelstein, 1983). Prehybridization and hybridization were performed according to Georges *et al.* (1988) using dried skimmed milk. Membranes were washed 2 × 15 min in 2 × SSC, 0.1 per cent SDS at room temperature, 2 × 15 min in 1.5 × SSC, 0.1 per cent SDS at +60°C and finally 2 × 10 min in 1 × SSC at room temperature and exposed to X-ray film (Kodak X-omat AR) at −70°C for 1–6 days with intensifying screens. Most membranes were subjected to different exposure times to visualize bands of different intensities. The DNA probe was removed by washing in 0.4 M NaOH and 0.2 M Tris, pH 7.5, checked for remaining radioactivity and then used for rehybridization.

Analysis

Karyotypes of mother and offspring were analysed according to Searle (1990). The karyotypic and fingerprint analyses were performed independently and results were not compared until both were complete.

The DNA fingerprint analysis was based only on information from the mother and her offspring as we know nothing about the fingerprints of the possible fathers. The male bands could be inferred by comparing the presence of bands in the female with all her offspring. The average identity between female-offspring is about 65 per cent, which gives an estimated mean identity between unrelated individuals of 0.4. The bandsharing of unrelated individuals is the probability that a band is present in a diploid zygote ($x = 0.4$) while q, the probability that a given band is present in a random gamete can be calculated from the Hardy–Weinberg formula ($q = 1 - \sqrt{1 - x}$). We assume that q is the same for all bands, in this case $q = 0.225$.

Assuming that there is no mutation, no linkage, or allelism and that all bands have the same population frequency of q, it is possible to calculate the probabilities associated with the distribution of different combinations of paternal alleles between offspring (a data set) given alternative paternity hypotheses. The appropriate way to calculate the probability of obtaining the observed dataset is to compare two alternative hypotheses, that the offspring have a single father, and there are two fathers. These individual probabilities will both

be very low, but the important statistic is their ratio, the likelihood ratio.

In the single father hypothesis, a paternal-specific band, found in some of the offspring but not others, must represent a heterozygous locus in that single father. The total probability of the data for that band is $2q(1-q)\,(1/2)^n$. The first part of the equation is the Hardy–Weinberg probability that the father is a heterozygote for the band, and the second part is the probability of the precise distribution of the band seen in the n offspring. The total probability of the dataset under this hypothesis is the product of the probabilities for all the paternal-specific bands. It is possible to calculate a corresponding probability for the hypothesis in which there are two fathers. It must be remembered, however, that there are $2^{n-1}-1$ different two-father hypothesis, which differ in the distribution of the two fathers across the offspring. It is illegitimate simply to take the most likely of these and compare it with the one-father hypothesis. Rather, the mean probability of obtaining the data across all these two-father hypotheses must be used. For more details of the methods used, see Brookfield (1989).

Results and discussion

Karyotypic analysis

The karyotypic data provide no definitive evidence for multiple paternity among the litters analysed. In fact, based on our knowledge of Robertsonian karyotypic variation in the Oxford area, this is not surprising. Among those pregnant females caught, only the single individual from East Hendred (2087) is from the interracial hybrid zone where certain chromosomes segregate for three 'alleles' (three different arrangements of the same chromosome arm: Searle, 1990). At Cothill, the other site sampled, all chromosomal polymorphisms are of the two 'allele' type.

In the litter of female 2103, equal numbers of heterozygous and homozygous offspring were expected for all three variable chromosomes if there was only a single sire. For one variable chromosome (*no*), four out of five offspring were heterozygous, for another (*pr*) a different four offspring were homozygous, while for a third variable chromosome (*kq*) segregation was as expected (three homozygotes: two heterozygotes). Thus, there is a slightly distorted segregation which may be the result of multiple paternity but the evidence is weak.

A more unusual segregation was observed in the litter of female 2087. Considering the only chromosome which varied among the litter (*no*), equal numbers of heterozygous and homozygous offspring were expected if there was only a single sire. In fact, six individuals were homozygous and only one individual (offspring 7) was heterozygous and showed a different karyotype from the other offspring. There was an additional peculiarity about the litter of female 2087. One of the two 'alleles' of chromosome *pr*, the metacentric form of the chromosome, which is rather less common than the alternative twin-acrocentric form at East Hendred (Searle, 1986) where the female was caught, was transmitted on the male side to all offspring. However, all one can say from the distorted segregation is that there is likely to be multiple paternity and off-spring 7 is the most likely to have a different father.

There is no indication of multiple paternity in litter 2102 as the female was heterozygous for all variable chromosomes and only four offspring were karyotyped, which minimizes the likelihood of detecting multiple paternity. There was no indication of multiple paternity in litter 2098 from karyotypic data. Female 2097 and her offspring were not karyotyped.

DNA fingerprint analysis

The average number of bands identified by the two probes in females is 19.4 where probe 33.15 often gives a slightly higher number. Among offspring, the mean total number of bands per individual detected by the two probes is 21.1 but the variation in band number per individual is considerable (range 14–28, Table 1). Comparisons of the same nylon membrane for whether the two probes detect identical fragments were negative and we therefore consider information from probes M13 and 33.15 to be independent.

The number of male-specific bands reconstructed from all offspring is 10 and 15 in the litters of females 2098 and 2102 respectively (two probes), 7 and 8 for litter 2103 and 2097 (only probe 33.15) and as high as 29 (two probes) for the offspring of female 2087 (Table 1).

The total number of male bands identified divided by the average number of bands within a litter would be expected to be slightly less than 60 per cent $(1-x)$. The ratio seen is close to this for litters 2103, 2098, and 2102. The higher figure (71 per cent) for litter 2097 is probably explained by random band segregation and by the fact that we used only one probe. This litter may, however, have been sired by a father with an unusual fingerprint pattern. In litter 2087, the ratio between the total number of male-specific bands and the average number of bands is very high (123 per cent, Table 1). The high number of male-specific bands (29) is evidence that more than one male has been involved in siring this litter.

Table 1 The number of offspring and male-specific bands identified by the two probes used for the investigation of multiple paternity in five wild-caught female common shrews (*Sorex araneus*)

Female	Number of offspring	Average number of bands in offspring				Total	Range	Number of male-specific bands		Number of male-specific bands per individual		Total number of male bands/average number of bands per offspring
		M13	Range	33.15	Range			M13	33.15	M13	33.15	
2087	7	12.3	9–15	11.2	7–14	23.5	16–28	14	15	5.5	6.0	29/23.5 = 1.23
2097	10	6.7	4–10	11.3	9–12	18.0	14–21	—	8	—	5.4	8/11.3 = 0.71
2098	6	8.7	6–11	9.0	7–10	17.7	16–21	6	4	2.2	1.3	10/17.7 = 0.56
2102	4	11.3	10–13	14.0	12–17	25.3	22–28	11	4	4.3	1.8	15/25.3 = 0.59
2103	4	—	—	14.5	12–17	—	—	—	7	—	4.0	7/14.5 = 0.48
Average	6.2	9.7		12.0		21.1		10.3	7.4	4.0	3.7	

Distribution of male-specific bands

DNA fingerprinting showed no indication of multiple paternity in the offspring of females 2098 and 2097. The offspring of female 2103 was probed only with 33.15 and seven male-specific bands were found. Two of these were found in all individuals, two bands were found in one individual only. There are seven two-father models to be compared to the one-father model. The dataset is more likely with a one-father model than with a two-father model and the indication of multiple paternity by the karyotypic data is not confirmed.

In the litter of female 2102 (Table 2), offspring number 1 shows five male-specific bands not shown by the litter mates having five different male-specific bands with probe M13. This would be expected if one male had sired one individual and another male the other three offspring. Probe 33.15 shows very few male-specific bands (4). There are seven two-father models (with a 3:1 or a 2:2 split among the offspring) and the mean of the seven likelihood ratios and one (representing the one-father model) is 6.5×10^4, which indicates that the distribution of male-specific bands between offspring is much more likely if there are two fathers. Given that there are two fathers, there was a 97.8 per cent chance that offspring 1 had a different father from offspring 2, 3 and 4. This case of apparent multiple paternity was not indicated by the karyotypic data.

Table 2 Distribution of male-specific bands identified by the two probes M13 and 33.15 in the litter of wild-caught female 2102 where multiple paternity is suggested

Male-specific band number	Male A	Male B		
	1	2	3	4
Probe M13				
1				x
2			x	
3		x		x
4	x			x
5	x			
6	x			
7	x			
8	x			
9		x	x	x
10	x			
11		x	x	x
Number of male bands	6	3	3	5
Probe 33.15				
1	x			x
2	x		x	x
3	x			
4				x
Number of male bands	3	0	1	3

In the litter of female 2087 there were 29 male-specific bands (Table 3). Five were found in one individual only, seven bands were found randomly distributed between offspring and could have been present in both male A and B (as expected from the average band-sharing between individuals). Seventeen out of 29 male bands had a non-random distribution between offspring. Thus, four M13 and four 33.15 male-specific bands characterize offspring 1, 3, 4 and 7 while another nine male-specific bands characterize offspring 2, 5 and 6, which suggests multiple paternity, with two fathers (A and B) siring four and three offspring, respectively. The arithmetic mean of the likelihood ratios for 63 possible models is 3.36×10^{15} with virtually all the probability derived from a model where

Table 3 Distribution of male-specific bands identified by the two probes M13 and 33.15 in the litter of wild-caught female 2087 where multiple paternity is suggested

Male-specific band number	Male A				Male B		
	1	3	4	7	2	5	6
Probe M13							
1—			x				
2*		x	x		x	x	
3A	x	x	x				
4B					x		x
5B					x	x	x
6B					x	x	x
7—				x			
8A	x	x		x			
9—							x
10A	x	x	x				
11B					x	x	x
12*	x				x		
13A	x	x					
14B					x		x
Number of male bands	5	5	4	2	7	4	6
Probe 33.15							
1A	x	x	x				
2B					x	x	x
3B					x	x	x
4A	x		x	x			
5*	x				x		
6*	x				x		
7A		x	x				
8B						x	x
9A		x	x				
10B						x	x
11*	x	x				x	x
12—				x			
13—	x						
14*		x			x	x	x
15*		x	x				x
Number of male bands	6	6	5	2	5	5	7

A and B = bands from putative males A and B respectively.
* = Bands present in both male A and B.
— = Non-informative band.

offspring 1, 3, 4 and 7 have a different father from the others. The extremely high value of this likelihood ratio constitutes strong evidence that this litter was sired by two fathers. Given that there were two fathers, there is a 99.99 per cent chance that the distribution of offspring above between fathers is correct. The karyotypic data indicated that offspring 7 may have a different father compared to some or all of the other offspring. This was also confirmed by the DNA-fingerprint analysis.

We have not detected any cases of linkage as variable female bands showed Mendelian inheritance in the litters examined. An alternative explanation to multiple paternity for the association of bands in offspring would be linkage, which can be tested. If there was only one father for all offspring, and he was heterozygous for the loci studied, on average half of the offspring would have the band and the other half would not. For litter 2087, with seven offspring, we would expect that each band on average would be found in 3.5 offspring. If we find that the representation of the bands among offspring is lower than this, then this constitutes evidence for double paternity which is independent of the evidence given above, and which cannot be the result of genetic linkage. For female 2087 each of the 29 male bands are found in, on average, 2.41 of the offspring, which is considerably less than the 3.5 expected. For the two-father hypothesis with offspring 2, 5 and 6 showing 11 male bands, each of these are found in, on average, 1.55 offspring compared to the 1.5 expected. For offspring 1, 3, 4 and 7 the 19 male bands are found in, on average, 1.84 of the offspring, which is close to the 2.0 expected. Thus, linkage is an unlikely explanation for the band distribution in litter 2087. The data for litter 2102, treated in the same way, are less convincing and do not resolve the issue for this litter but indicate the right direction.

Conclusion

Thus, karyotypic data indicated, but did not provide, definitive evidence for multiple paternity in two litters and it was not possible to assign particular offspring to particular fathers. DNA-fingerprinting provided clear evidence for two fathers siring the offspring of two out of five females and offspring could be assigned to particular fathers. In his karyotypic study, Searle (1990) found that among the offspring from 16 wild-caught female common shrews, there were four litters for which it was possible to demonstrate multiple paternity. In chromosomal analysis, multiple paternity can be detected only when sires of a particular litter happen to contribute three different chromosomal arrangements of the same chromosome arm, which is a common situation only in contact zones between chromosomal races. Even if the common shrew has one of the most variable karyotypes of any mammal (Wójcik & Searle, 1988), analysis of chromosomal variation is likely to underestimate the frequency of multiple paternity.

Despite the small numbers of individuals examined, the present study suggests that multiple paternity is not a rare phenomenon in the common shrew, that DNA-fingerprinting has the potential to reveal multiple

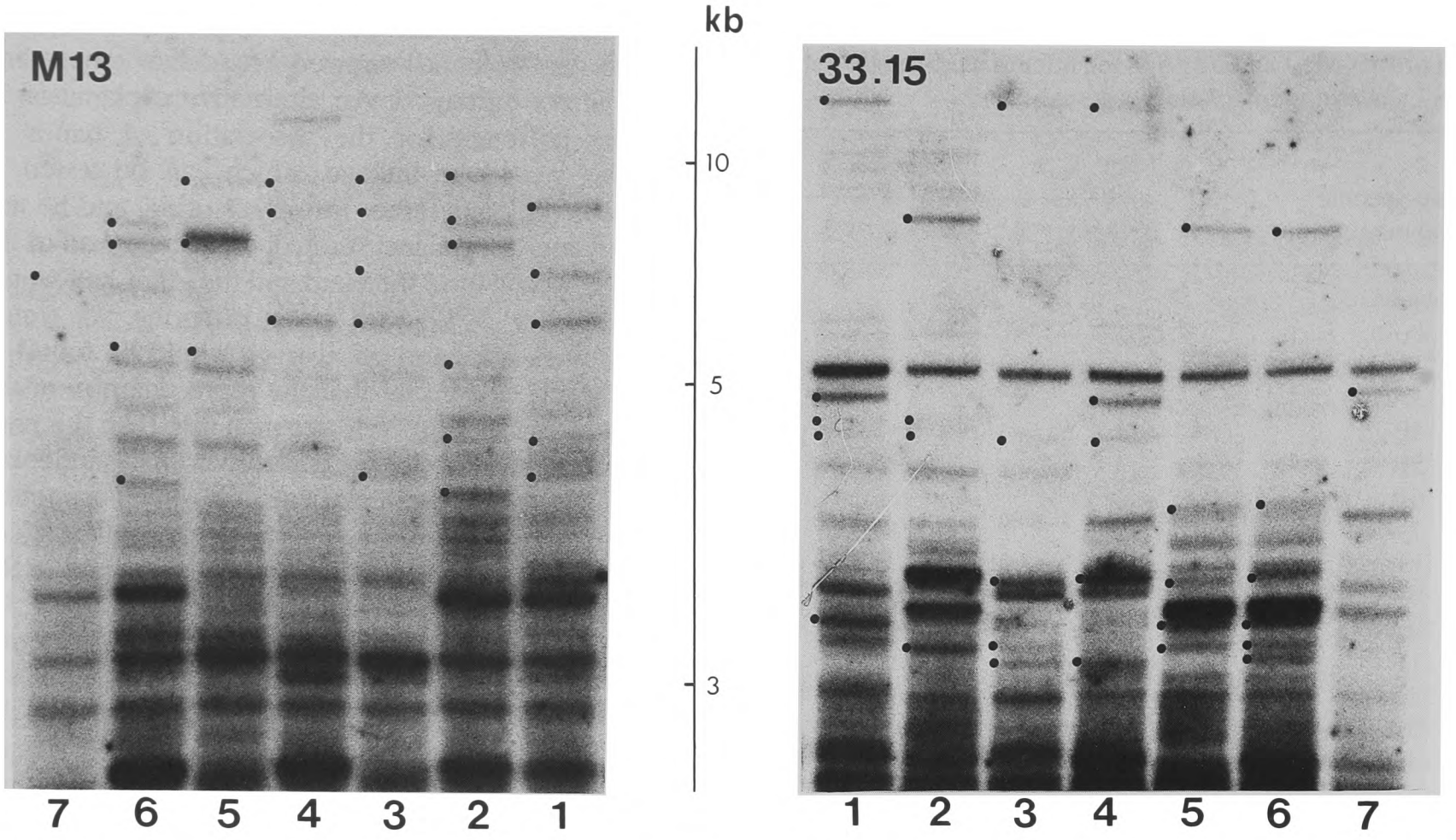

Fig. 1 DNA-fingerprints of the offspring (1–7) of female 2087 where multiple paternity is suggested. Informative male-specific bands used in the analysis are indicated (●).

paternity and that it is possible to assign individual offspring to different male parents even when none of the putative fathers are available for inspection. In common with other species of mammal, where the male does not contribute to rearing the offspring (Ginsberg & Huck, 1989), the mating system of the common shrew appears to be promiscuous. When multiple matings occur the promotion of sperm competition (Parker, 1970) may represent a female strategy and our understanding of such sperm manipulation is of great importance for the evaluation of mating strategies and social organization (Dewsbury & Baumgardner, 1981; Birkhead & Hunter, 1990).

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