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Parentage in the cooperative breeding system of long-tailed tits, *Aegithalos caudatus*

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In cooperatively breeding birds multiple maternity and paternity of broods is not uncommon, reproduction often being shared among group members as well as with extragroup members. We investigated the extent of extrapair paternity and intraspecific brood parasitism in a population of cooperatively breeding long-tailed tits. Our aim was to determine the frequency and cause of mixed parentage and to investigate whether shared maternity or paternity was associated with decisions made by helpers. Genetic analyses using eight microsatellite loci showed that extrapair paternity was low (2.4–6.9% of nestlings in 16–29% of broods), and that intraspecific brood parasitism was negligible. Mate switching and extrapair copulations were both observed, but mate switching was not responsible for the mixed paternity we recorded. Some extrapair offspring were assigned to males that became helpers at the nest containing their extrapair young, but these males were also close neighbours of the cuckolded males and so were the most likely males to gain extrapair paternity. There was no evidence that the existence of a direct reproductive stake in a brood played an important role in the helping decisions of either male or female helpers.

In cooperative breeding systems more than two individuals cooperate to rear a single brood (Brown 1987). Cooperatively breeding species are typified by nuclear families that form through the delayed dispersal of grown offspring who then help their parents to rear subsequent broods (Stacey & Koenig 1990). In such species, helpers do not usually have any direct reproductive stake in the helped brood but are thought to accrue indirect or direct fitness benefits through their helping behaviour (for reviews see Emlen 1991, 1997; Cockburn 1998). Helper reproduction within nuclear families is likely to be constrained by inbreeding avoidance (Ralls et al. 1986; Pusey & Wolf 1996; but see Reeve et al. 1990; Keane et al. 1996; McRae 1996), but there may be opportunities for extragroup copulations by breeders and helpers. For example, in the Maluridae the majority of chicks are fathered by extragroup males (Brooker et al. 1990; Double & Cockburn 2000).

Some cooperatively breeding species have more complex social and reproductive strategies in which reproduction is shared among group members (Emlen 1991). In cooperative polyandry, two or more males in a group may share paternity of a brood belonging to a single female (e.g. Burke et al. 1989; Jamieson et al. 1994; Faaborg et al. 1995) and, in joint-nesting species, mixed maternity may result when two or more females lay eggs in a single nest (e.g. Koenig et al. 1995; McRae 1996; Jamieson 1997). Variation in the extent of reproductive sharing is also evident within species: the breeding system of a single species may encompass simple monogamy, helpers-at-the-nest, shared paternity or shared maternity (e.g. Koenig & Mumme 1987; Whittingham et al. 1997; Richardson et al. 2001).

The ultimate basis for this rich array of reproductive strategies among cooperatively breeding vertebrates is currently poorly understood. The application of optimal reproductive skew theory to the sharing of reproduction in animal societies has generated a substantial body of theory but has met with mixed success in explaining the diversity of reproductive strategies in social vertebrates (Vehrencamp 1983, 2000; Keller & Reeve 1994; Clutton-Brock 1998; Reeve et al. 1998; Magrath & Heinsohn 2000). What is clear is that the functional significance of helping behaviour can be fully understood only when the extent of reproductive sharing in a given species or population has been determined. For example, Cockburn (1998) reviewed the literature on direct reproduction by

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Table 1. Polymorphic loci used in DNA profiling for parentage analysis of long-tailed tits, showing the primer label, annealing temperature T_a and $MgCl_2$ concentration, the number and size range of alleles, and the exclusion probabilities for one parent (Exclusion 1) and a second parent when the first is known (Exclusion 2) for each locus

						Exclusion	
Locus	Primer label	T_a (°C)	$MgCl_2$ (mM)	Alleles	Size range	1	2
*Ase*37	6-FAM R	60.0	1.0	16	236–279	0.557	0.717
*Esc*6	6-FAM R	51.0	1.0	4	138–144	0.158	0.296
*Hru*2	NED R	51.0	0.6	6	132–142	0.220	0.398
*Hru*6	HEX F	55.0	2.0	36	240–330	0.792	0.883
*Lox*1	NED R	51.0	1.0	11	257–282	0.360	0.541
*Mme*8	6-FAM F	50.5	1.0	3	198–212	0.029	0.124
*Pca*3	HEX R	60.0	2.0	6	149–159	0.321	0.496
*Pca*4	6-FAM R	58.0	1.0	10	160–178	0.391	0.570
				Mean=11.5		Total=0.985	0.999

Data refer to results from 711 individuals. References: *Ase*37: Richardson et al. (2000); *Esc*6: Hanotte et al. (1994); *Hru*2, *Hru*6: Primmer et al. (1995); *Lox*1: Piertney et al. (1999); *Mme*8: Jeffery et al. (2001); *Pca*3, *Pca*4: Dawson et al. (2000).

helpers and suggested that it has previously been underestimated and that, consequently, the role of kin selection in the evolution of cooperative breeding systems has been overestimated.

Our aim in this study was to describe patterns of parentage in the cooperatively breeding long-tailed tit. We used DNA profiling to determine the frequency of extrapair paternity and intraspecific brood parasitism. Behavioural observations were used to investigate the likely source of mixed parentage within broods and to determine whether parentage plays an important role in the caring decisions of helpers.

METHODS

Study Species and Population

Long-tailed tits spend the nonbreeding season in flocks that include both relatives and nonrelatives (Hatchwell et al. 2001a). Flocks break up in the early spring, males adopt part of the winter range as their breeding range and pair either with unrelated females from the same flock or with immigrant females. There is an even adult sex ratio and all members of the population attempt initially to breed independently in pairs, females laying a clutch of 8–11 eggs. Nest failure is frequent (Hatchwell et al. 1999) and individuals whose nesting attempt fails after early May may become helpers at the nest of another pair, assisting them in feeding nestlings and fledglings (Glen & Perrins 1988). As a consequence of the large number of failed breeders, about half of all broods in our study population have helpers (range 1–4) during the nestling period. Helpers allow parents to reduce their provisioning rates (Hatchwell & Russell 1996; Hatchwell 1999) and also increase the condition and subsequent recruitment of offspring (Glen & Perrins 1988; Russell 1999; B. J. Hatchwell, unpublished data).

We studied a population of 14–53 pairs of long-tailed tits annually from 1994 to 2000 in the Rivelin Valley, Sheffield, U.K. We closely monitored the breeding attempts of all pairs in the study site throughout each breeding season (March to June). Immigrants were usually colour ringed before egg laying started, so the majority of birds were individually marked before breeding in each year (males 85–100%, females 78–98%). We located the majority of nests before egg laying and established the identity of pair members through observations of nest building. Once the eggs had hatched, we observed nests, usually for 1 h, on alternate days from hatching (day 0) to fledging (day 16 or 17), or until the nest was depredated. We recorded the identity of each bird provisioning nestlings. We individually colour-ringed 11-day-old nestlings, took a blood sample of 10–50 μl by brachial venipuncture (under Home Office licence) and immediately mixed it with 98% ethanol. A tissue sample was taken from any recently dead nestlings present in the nest and stored in ethanol. Adults were blood-sampled (10–50 μl) by brachial venipuncture at the time of ringing.

DNA Extraction and Analysis

We extracted DNA from blood or tissue, using phenol extraction (Bruford et al. 1998). We dissolved DNA samples in 1 × TE (10 mM Tris, 0.1 mM EDTA) and determined their concentration with a DyNAquant 200 fluorometer. Samples were then diluted to a concentration of 10–50 ng/μl.

We screened DNA samples with 73 microsatellite markers, 56 of which gave a product. Nine loci were polymorphic in a sample of 32 unrelated individuals, producing at least three alleles, and we selected eight of these on the basis of polymorphism and product size for use in parentage analysis (Table 1). For DNA reactions we used a 10-μl volume containing 10–50 ng of DNA, 1.0 μM of each primer, 0.2 mM of each dNTP, 0.25 units of Taq DNA polymerase (Thermoprime Plus, ABgene, Epsom, U.K.) and 0.6–2.0 mM $MgCl_2$ (Table 1) in 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl pH 9.0, 0.01% (w/v) Tween. For PCR amplification we used Hybaid Touchdown thermal cycler. The reaction profile was 94°C for 3 min, then 94°C for 30 s, X°C for 30 s, 72°C for 30 s for 35 cycles (where X varied from 50.5 to 60.0°C, Table 1), followed by 72°C for 10 min. For one of the primers the 5′ end was labelled with a fluorescent phosphoramidite (6-FAM, HEX or NED) and the other primer was PIG-tailed to reduce noise (Brownstein et al. 1996; Table 1).

PCR products were visualized on an ABI 377 automated DNA sequencer, using an internal size standard (ROX500). The combination of the three dyes and non-overlapping size ranges of the markers (Table 1) allowed us to load all the PCR products for an individual in a single lane. To analyse the gels we used ABI GeneScan (version 3.1) and Genotyper (version 2.5) DNA fragment analysis software. The overall exclusionary power for the loci used in parentage analyses was 0.985 for the first parent and 0.999 for the second (Table 1).

Parentage Analysis

To analyse genotype data we used Cervus (Marshall et al. 1998), a software package that uses a likelihood-based approach to infer parentage. The natural logarithm of the likelihood ratio is termed the LOD score. A LOD score of zero implies that the putative father is as likely to be the father as a randomly chosen male. A positive LOD score implies that the male is more likely to be the father than a randomly chosen male (Marshall et al. 1998). Cervus simulates the critical difference in LOD scores between candidate parents for assignment at chosen levels of confidence. We assumed a 1% error rate in typing and that 95% of candidate parents were sampled. The delta criterion was between 0.32 and 0.78 in different years for 95% confidence and was 0.00 for 80% confidence where one parent was known (Marshall et al. 1998).

In long-tailed tits, helpers are usually present only during the nestling period, so the social unit during a female's fertile period is a monogamous pair. Therefore, we first used Cervus for an 'exclusion analysis', as would be conducted to detect extrapair paternity in a monogamous system. Putative mothers and fathers were those breeders that built and attended a nest throughout the breeding cycle. Second, in a 'group analysis', we included as candidate parents both the breeding pair and any birds that became helpers because one of the questions that we were addressing was whether helpers assist at nests where they have a direct reproductive stake, either through shared paternity (male helpers) or joint nesting (female helpers). Finally, in an 'open analysis' using Cervus, having assigned maternity in previous analyses, we included as candidate fathers all sampled males in the population that were alive in the year of the breeding attempt.

We assigned parentage in a series of steps. For complete families in which both putative parents were blood-sampled (N=296 nestlings in 39 broods), we first assigned maternity with unknown paternity. We then attempted to assign paternity to either the putative father, a male from the group, or a male from the population. The presence of close relatives to putative fathers and mothers in both group and open Cervus analyses of long-tailed tit parentage presents a potential problem for assignment of parentage. Thompson & Meagher (1987) pointed out that if offspring have full siblings in the population, and maternity is unknown, the full sibling will, on average, have a higher probability of paternity than the true father. However, Marshall et al. (1998) argued that confidence of assignment is only marginally affected by the presence in the population of close relatives of one of the true parents once the first parent has been assigned. Furthermore, pairs of long-tailed tits that breed successfully usually divorce between breeding attempts (Hatchwell et al. 2000), so broodmates are usually the only full siblings of nestlings in the population. Nevertheless, the paternity of some maternally assigned offspring from our population remained ambiguous following Cervus analysis (see Results). Given that relative LOD scores may not reflect actual parentage under such circumstances (Thompson & Meagher 1987), we used additional criteria for assignment of paternity (see Results).

For some families, a blood sample was available for just one putative parent. If the putative mother was sampled, the procedure described above was followed (maternity assignment represents the first step in the assignment sequence) to determine whether the putative mother was the true mother. For families from which the putative father alone had been sampled we again attempted to assign paternity using Cervus.

RESULTS

Parentage Analysis

We obtained LOD scores from Cervus for parents of 296 offspring from 39 complete families. A further 73 offspring from 12 broods were compared with their putative mothers only and 84 offspring from 10 broods with their putative father only. We assigned 98.9% (365/369) of offspring to the female breeder with a high degree of confidence (P>0.95). The putative mother was excluded for just four nestlings, all from a single brood of eight chicks. Three of these were assigned to a second female that was known to have laid eggs in the same nest but the fourth could not be assigned to either female seen attending the nest, nor to any other sampled female in the population. This brood resulted from an unusual series of events, described in 'Joint nesting' below.

We then attempted to assign paternity for the 288 maternally assigned offspring from 38 complete families (excluding the brood with multiple maternity). In the exclusion analysis, the putative father was excluded for seven of 288 (2.4%) offspring in six of 38 (15.8%) broods, and assigned with a confidence of P>0.80 for the remaining offspring. For the 10 families (84 nestlings) from which the putative father alone had been sampled, no putative fathers were excluded and all offspring were assigned with a confidence of P>0.80. Thus, the putative father was excluded for seven of 372 (1.9%) nestlings in six of 48 (12.5%) broods. However, given the problems of paternity assignment using Cervus with a limited set of loci in kin-structured populations, coupled with the relatively low probability of correct assignment of the true father when the first parent has not been assigned (Marshall et al. 1998), we made no further attempt to resolve paternity of these male-only broods.

In the 'group analysis', including those males that attended a brood (male breeder plus any male helpers) as candidate fathers, 267 of 288 (92.7%) offspring were assigned with at least 80% confidence to the putative father, 17 of 288 (5.9%) offspring in nine of 38 (23.7%) broods were assigned to male helpers, leaving just four of 288 (1.4%) offspring in four broods unassigned.

Finally, in the 'open analysis', 240 of 288 (83.3%) offspring were assigned with at least 80% confidence to the putative father. These offspring were also assigned to the putative father in the exclusion and group analyses, and can be regarded as unequivocal within-pair offspring. Of the remaining 48 offspring, 27 were assigned to the putative father in the exclusion and group analyses but to an extragroup male in the open analysis, while 17 were assigned to an extrapair male in both group and open analyses. All four offspring that were not assigned in the exclusion and group analyses were assigned to extrapair males in the open analysis.

There is clearly some disparity in paternity assignments by Cervus according to the number and identity of candidate males: estimates for extrapair paternity (EPP) in complete families ranged from 2.4 to 16.7% of chicks in 15.8 to 60.5% of broods. However, paternity assignment in kin-structured populations presents particular problems (Marshall et al. 1998). For example, in the open analysis, the putative father was identified by Cervus as the second most likely father in 26 of 48 (54%) cases where he was not the assigned male. The inconsistent assignment of paternity in the three analyses results from overlapping LOD score distributions. The LOD scores of unequivocal within-pair offspring (i.e. assigned to putative father in all analyses) are all positive and greater than 1 (Fig. 1a), but this is also true of the majority of the 48 potentially extrapair offspring and their putative fathers (Fig. 1b), even though they were assigned to an extrapair male in the open analysis. Similarly, these 48 offspring all had positive LOD scores with the assigned extrapair fathers (Fig. 1c), the distribution of which overlapped that of assigned putative fathers.

The spatial distribution of assigned extrapair fathers in the open analysis suggests that many of them were unlikely to be true fathers of sampled offspring. The 31 extrapair males assigned as fathers of 48 offspring in the open analysis occupied ranges up to 2.6 km from the nest ($\bar{X} \pm SD=995 \pm 700$ m, $N=31$; Fig. 2a), while all of the assigned extrapair fathers of chicks whose putative fathers were unequivocally excluded occupied ranges relatively close to the nest (325 ± 243 m, $N=7$; Mann–Whitney U test: $Z=2.69$, $P=0.007$; Fig. 2a). Figure 2b shows the distribution of internest distances for the first nests of all males in the study population in a year of average density (1999, $N=41$ pairs); this represents the expected distribution of internest distances if extrapair paternity were assigned randomly among males in the population. There was a highly significant difference between this random distribution of internest distances ($\bar{X} \pm SD=1111 \pm 644$ m, $N=820$) and the distribution of unequivocally assigned extrapair fathers (Fig. 2a; Mann–Whitney U test: $Z=3.56$, $P<0.001$). By contrast, there was no significant difference between the expected internest distribution for the population and the inter-nest distribution for assigned extrapair fathers in the open Cervus analysis (Fig. 2a; Mann–Whitney U test: $Z=1.14$, $P=0.25$). Closely related males (coefficient of relatedness, $r=0.5$) had relatively short internest distances ($\bar{X} \pm SD=255 \pm 129$ m, $N=35$; Fig. 2b), so that neighbouring males were more likely to be closely related than distant males. This illustrates the difficulty of assigning paternity in kin-structured populations.

These comparisons suggest that the majority of the equivocally assigned extrapair fathers in the open Cervus analysis were unlikely to have been the true fathers; a more realistic number of extrapair males can be estimated as follows. If we assume that the spatial distribution of extrapair males that were assigned following the exclusion analysis is the 'correct' distribution of extrapair males with respect to nest location, and if all six of the assigned males from the open analysis within 300 m (Fig. 2a) are correctly assigned extrapair fathers, we can calculate the proportion of more distant extrapair males expected to have been correctly assigned. By this reasoning, 2.4 (i.e. [6/5] × 2 males; see Fig. 2a) of the 10 assigned males from the open analysis between 300 and 900 m would be expected to be extrapair males, giving approximately eight additional extrapair fathers. The eight closest males from the open analysis were assigned as extrapair fathers of 13 chicks in six broods. Adding these to the seven unequivocal cases gives a total of 20 of 288 (6.9%) extrapair nestlings in 11 of 38 (28.9%) broods. However, we emphasize that this is probably a generous estimate because of the assumption that all assigned males within 300 m were indeed extrapair fathers. In addition for 10 of these 13 'extrapair' offspring the pair male had a LOD score >1 ($\bar{X} \pm SD=+2.54 \pm 0.96$, $N=10$), that is above the threshold for unequivocal within-pair offspring (Fig. 1a).

In summary, we conclude that extrapair paternity in long-tailed tits is in the region of 2.4 (95% CI 0.8–4.3%) to 6.9% (4.4–10.5%) of nestlings, in 15.8 (6.1–30.5%) to 28.9% (15.7–44.3%) of broods, and most probably towards the bottom of this range.

Mate Switching

Mate switches involved both males and females. In 213 'female-years', 8.9% of females were known to have breeding attempts with two males within a season, a mean of 1.09 partners/female per season. For males, 7.4% ($N=231$ 'male-years') bred with two or more females within a season, a mean of 1.08 partners/male per season. Here, we focus on females because of the consequences that multiple mating by females might have for the paternity of their broods. The majority of mate switches occurred immediately after one breeding attempt failed and before initiation of the next, that is about 8 days before the start of laying (Fig. 3). In just two of 15 (13%) cases did a female switch partners during egg laying

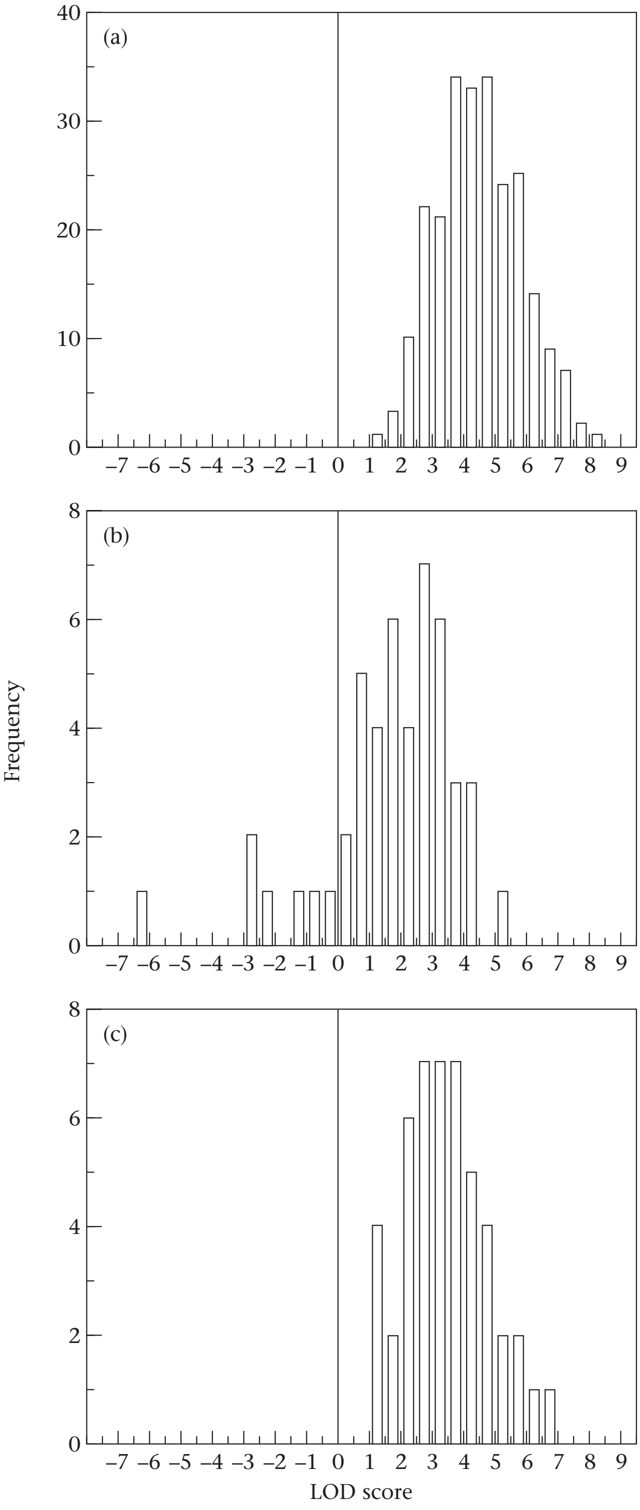

Figure 1. LOD score distributions from Cervus analysis of paternity in long-tailed tits. A positive LOD score (to the right of the vertical line) implies that a male is more likely to be the father than a randomly chosen male from the population. (a) LOD scores for offspring that were unequivocally assigned to the pair male in all Cervus analyses (*N*=240); (b) LOD scores of pair males with offspring assigned to extrapair males in the Cervus analysis that included all sampled males in the population as candidate fathers, the 'open' analysis (*N*=48); and (c) LOD scores of extrapair males with offspring that were assigned to them in the open analysis (*N*=48).

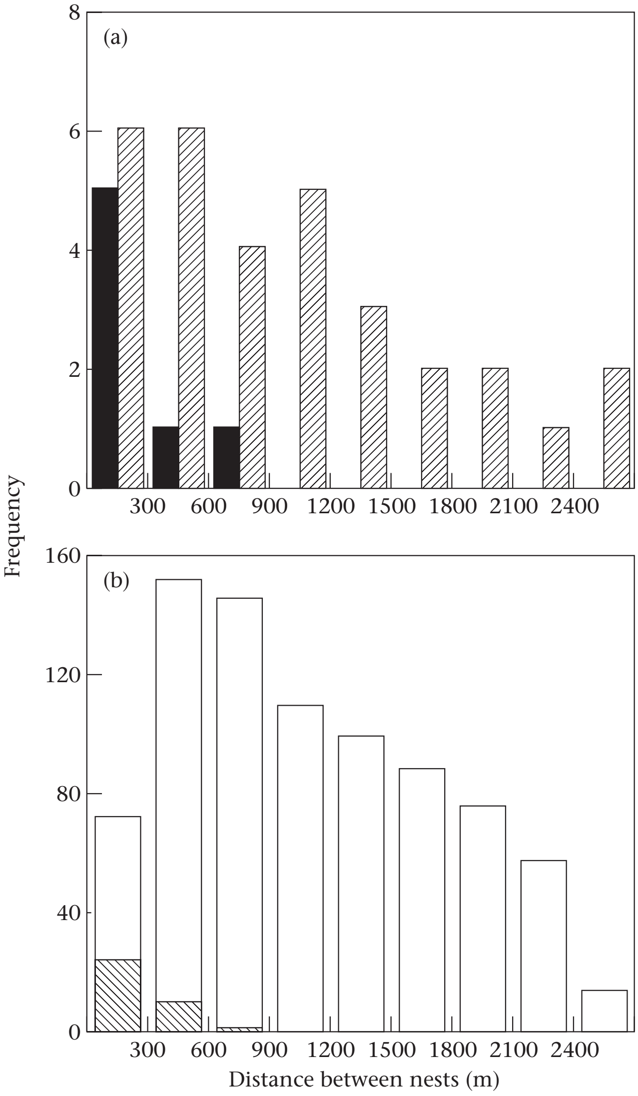

Figure 2. Frequency distributions of: (a) distances between nests of offspring and extrapair males (*N*=7) assigned as fathers of seven nestlings for which the putative father was unequivocally excluded (■), and distances between nests of offspring and extrapair males (*N*=31) assigned as father of 48 nestlings that were not unequivocally assigned to their putative father (▨); and (b) internest distances for all first nests of distantly related or unrelated males (*N*=785, ☐) and closely related males (*N*=35, ▧) in 41 pairs in 1999, a year of average breeding density.

(Fig. 3). The majority of breeding attempts that followed a mate switch were depredated before offspring were sampled, but we determined parentage of two broods: in both cases the switch occurred before egg laying started and the second male was the assigned father. Thus, we documented no cases where mate switching resulted in mixed paternity.

Joint Nesting

We observed two instances of more than one female laying in the same nest. In one case a female laid a clutch of 10 eggs, but disappeared before the start of incubation.

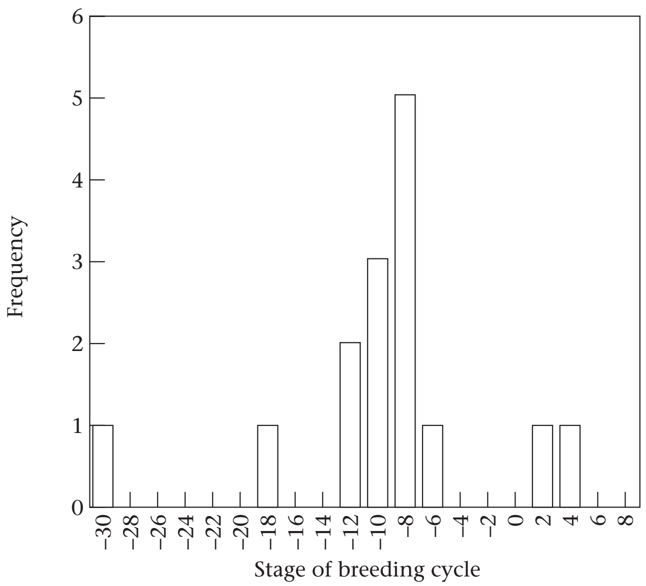

Figure 3. Timing of within-season mate switches by long-tailed tits relative to the prelaying and laying periods (day 0=first egg lay date).

An unrelated pair reused this nest, initiating a second clutch 21 days after completion of the first. There were eight nestlings on day 11 of the subsequent nestling period; seven were confidently assigned ($P>0.95$) to either the first ($N=3$) or the second pair ($N=4$) and one could not be assigned to any bird from our study population. We conclude that some eggs from both clutches hatched and that a third female dumped at least one egg in the nest. The second case of joint nesting involved a pair of failed breeders that became helpers. The female helper laid seven eggs in the nest alongside the assisted brood, but no eggs from the second clutch hatched. No other instances of joint nesting or intraspecific brood parasitism were recorded, observations consistent with assignment of maternity to the putative mother in 98.9% of cases (100% if the first case described above is excluded).

DISCUSSION

Our results showed a low level of extrapair paternity among long-tailed tit nestlings (2.4–6.9% of nestlings in 16–29% of broods). Mate switching was common but was not responsible for the mixed paternity that we recorded. Furthermore, mixed maternity was negligible, showing that intraspecific brood parasitism and joint nesting were rare in our study population.

The assignment of offspring to putative mothers was unequivocal for the majority of sampled nestlings. Using a molecular sexing technique (Griffiths et al. 1998) and behavioural observations, we sexed 86 helpers, 12.8% of which were females (B. J. Hatchwell, unpublished data). None of these female helpers had a direct reproductive stake in the brood they helped, although one laid eggs in a nest while she helped provision the brood. Therefore, although joint nesting is an important reproductive strategy in several cooperatively breeding species (e.g. Vehrencamp 1978, 2000; Koenig & Mumme 1987; Bertram 1992; McRae 1996; Richardson et al. 2001), this is not the case in long-tailed tits. The single brood of mixed maternity resulted from clutches laid sequentially by two females in the same nest. This case is remarkable not only because it is clearly unusual behaviour in this species, but also because some eggs from the first clutch remained viable despite not being incubated for at least 30 days. It is unlikely that embryo development would start at ambient temperature during this interval, but a preincubation interval of this length is at the limit of viability for chicken eggs at ambient temperature (Romanoff 1960).

Putative fathers were excluded for just 2.4% of nestlings in 15.8% of broods. Inclusion analyses gave higher values of EPP, although the ‘true’ value is probably considerably lower than the 16.7% suggested in the open analysis. We believe that a more realistic upper limit is 6.9% of nestlings in 28.9% of broods and even this is likely to be a considerable overestimate. Male long-tailed tits often have close relatives that are near neighbours in the same population (Fig. 2b; indeed this represents the basis for their cooperative breeding system, Russell & Hatchwell 2001), and the close kinship of candidate fathers creates problems for paternity assignment. Despite these difficulties, it is clear that the key result of the paternity analysis is that the rate of EPP in long-tailed tits is low, with the 95% CI for our upper estimate being just 10.5% of nestlings.

Extrapair offspring could have resulted from either mate switching or extrapair copulation (EPC). The majority of cooperative breeders have high mate fidelity (e.g. Stacey & Koenig 1990; Russell & Rowley 1996) a trait that is often associated with limited dispersal and high longevity (Ens et al. 1996); these are characteristic features of cooperative species (Brown 1987; Arnold & Owens 1998). Long-tailed tits are unusual among cooperative breeders in having a high rate of divorce between breeding seasons (Hatchwell et al. 2000), as well as the mate switches described here. However, mate switching did not result in multiple parentage in this study. Furthermore, in all broods where mixed paternity was known or likely, the putative parents were known to have been paired throughout the nest-building and laying periods. Therefore, we reject mate switching as a source of multiple paternity in sampled broods. This is not to say that mate switching never results in EPP; switches sometimes occurred during the laying period and could have led to multiple paternity of broods had the brood survived.

It is more likely that EPP in long-tailed tit broods resulted from EPC. We observed just nine copulations in 7 years: eight pair copulations and one EPC. All pair copulations were between day −2 and day 8 (where day 0=day of first egg), that is within the probable fertile period of the females involved. The single EPC was initiated by a male and occurred on the day of clutch completion, and so could not have fertilized any eggs. The relatively small testis size of long-tailed tits (T. R. Birkhead, personal communication) is consistent with a low copulation and EPP rate (Møller & Briskie 1995).

No general prediction can be made regarding the frequency of EPP in cooperative species because the selective pressures on the mating strategies of males and females vary, particularly in relation to the relative importance of direct and indirect fitness benefits in the evolution or maintenance of cooperation. Therefore, it is not surprising that Cockburn (1998) showed that the EPP rate among cooperatively breeding birds is highly variable, being absent in some species and reaching remarkably high levels in others, such as in the Maluridae (Brooker et al. 1990; Double & Cockburn 2000).

Male provisioning of nestlings is related to their paternity of a brood in several cooperatively breeding species, such as dunnocks, *Prunella modularis* (Burke et al. 1989; Davies et al. 1992), alpine accentors, *Prunella collaris* (Hartley et al. 1995), and white-browed scrubwrens, *Sericornis frontalis* (Magrath & Whittingham 1997; Whittingham et al. 1997; Whittingham & Dunn 1998). Females may therefore be able to recruit males to care for their brood by seeking copulations with potential carers. However, in cooperative breeding systems where groups are composed primarily of kin and cooperation is based on collateral kinship rather than shared paternity, helper reproduction within a group may be constrained by inbreeding avoidance. The majority of long-tailed tit helpers are male (87.2% of 86 sexed helpers) and they usually feed a brood belonging to a closely related male; by contrast, 70% of male helpers are unrelated to the female breeder (Russell & Hatchwell 2001). Therefore, the majority of helpers are unconstrained by incest avoidance, raising the possibility that helping behaviour in long-tailed tits might be influenced by parentage, that is male helpers could gain direct fitness benefits through shared paternity of helped broods.

We regard this possibility as unlikely for several reasons. First, unlike the majority of cooperative breeders, during the female's fertile period long-tailed tit helpers are not closely associated with the pair they help (Russell 1999). Even in the confamilial bushtit *Psaltriparus minimus,* where helpers are often present during egg laying (Sloane 1996), pairs are sexually as well as socially monogamous (Bruce et al. 1996). Second, there is a large stochastic element to the identity of helpers and helped broods because these roles are determined primarily by the success or failure of breeding attempts. If direct reproduction by helpers played an important role in helping decisions, extensive 'sharing' of paternity among potential helpers and the potential recipients of their care would be required. Finally, and most importantly, we found little evidence that a share of paternity was required for males to become helpers. There was just one brood where a helper may have had a substantial direct reproductive stake, being assigned as father of six out of 10 offspring in the group and open analyses. Out of the 28 helpers that attended broods that were included in the Cervus analyses, just six (21%) were assigned as parents (P>0.80) of offspring in the helped brood. Using the upper estimate of 6.9% EPP from the open analysis, we found that 11 of 20 (55%) extrapair nestlings in six of 11 (54.5%) broods were assigned to a helper at the same nest. However, this probably overestimates helper paternity because in the exclusion analysis the pair male was excluded for just one of these 11 nestlings. Furthermore, a substantial proportion of any extrapair young would be expected to be fathered by helpers simply because a third of close neighbours are also close relatives (Fig. 2b), and it is these close relatives that are likely to become helpers (Russell & Hatchwell 2001).

Therefore, although a male helper was sometimes the father of a nestling in the brood he helped, there was no close association between male help and shared paternity of a brood. A direct reproductive stake in a brood is therefore not required for a male to become a helper. This conclusion is supported by the striking contrast between the low level of EPP (2.4–6.9%) and the high frequency of helping (ca. 50% of nests had one to four helpers in our study population). Any tendency for extrapair chicks to be fathered by helpers is probably a consequence of male philopatry. The absence of any substantial direct reproductive stake in helped broods, for either male or female helpers, indicates that cooperative behaviour in long-tailed tits must have evolved or be maintained by the accrual either of other direct fitness benefits or of indirect fitness benefits through kin selection (Hatchwell et al. 2001b; Russell & Hatchwell 2001). The relative importance of the direct and indirect components of inclusive fitness for the evolution of cooperation in this species is currently being investigated.

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