Parentage, reproductive success and breeding behaviour in the greater horseshoe bat (*Rhinolophus ferrumequinum*)

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Female greater horseshoe bats form maternity colonies each summer in order to give birth and raise young. During the mating period, females visit males occupying territorial sites, copulation takes place and sperm are stored until ovulation occurs, normally in April. Using microsatellite markers and a likelihood method of parentage analysis, we studied breeding behaviour and male reproductive success over a five-year period in a population of bats in south-west Britain. Paternity was assigned with 80% confidence to 44% of young born in five successive cohorts. While a small annual skew in male reproductive success was detected, the variance increased over five years due to the repeated success of a few individuals. Mating was polygynous, although some females gave birth to offspring sired by the same male in separate years. Such repeated partnerships probably result from fidelity for either mating sites or individuals or from sperm competition. Females mated with males born both within and outside their own natal colony; however, relatedness between parents was no less than the average recorded for male–female pairs. Gene flow between colonies is likely to be primarily mediated by both female and male dispersal during the mating period rather than more permanent movements.

Keywords: Chiroptera; *Rhinolophus ferrumequinum*; paternity; reproductive success; relatedness; microsatellites

1. INTRODUCTION

The genetic structure of populations is largely determined by the breeding behaviour of the individuals within them. In particular, a high variance in male reproductive success associated with polygyny can decrease the effective population size, potentially accelerating genetic drift and selection (e.g. Chesser 1991). When male reproductive success is determined by female choice, then female dispersal during mating will influence spatial differentiation, while the male reproductive life span will affect the extent of temporal differentiation. Detailed long-term studies of breeding behaviour can therefore provide important insights into the mechanisms underlying the apportionment and maintenance of genetic variation, which are particularly useful in endangered species where habitat management is undertaken. Although such studies have been conducted on several mammals, including ungulates, primates and pinnipeds (reviewed by Clutton-Brock 1988), as yet no investigation on bats has provided longitudinal data of breeding patterns and male reproductive success exceeding three years. In this paper, we describe, to the best of our knowledge, the most detailed study to date of parentage and breeding behaviour in a bat species and present data on individual male reproductive success monitored over five years.

The greater horseshoe bat (*Rhinolophus ferrumequinum*) is one of the best studied of all chiropterans. Females are philopatric, forming summer maternity colonies, while adult males are predominantly solitary, roosting mostly underground throughout the year (Ransome 1991).

Females usually begin to breed in their third year and, although they may not do so in some years, can continue to breed up to the age of 29 years (Ransome 1995). In Britain, single pups are born around July (Ransome & McOwat 1994), of which an average of 72% die before their second summer (Ransome 1990). While Ransome (1995) showed that lifetime reproductive success differs widely among females, nothing is known about the reproductive success of males. In autumn, females visit males to mate, although copulation can also occur in winter and spring and, therefore, breeding partnerships appear to be primarily determined by female choice. Matings take place in territory sites occupied by single males, which may be small caves, mines or cellars or a specific part of a larger underground system (Ransome 1991). During the autumn and spring, up to eight females have been observed to visit a single male (Ransome 1991). Ringing data have shown that some males repeatedly return to the same territory for up to 16 years and these are often revisited by the same females (R. D. Ransome, personal observation). Following insemination, a vaginal plug forms inside the female and sperm are stored until fertilization occurs in the spring (Matthews 1937). The plugs may serve to prevent further mating, thus potentially precluding sperm competition and so promoting reproductive skew. Observations therefore suggest that breeding is polygynous and that male reproductive success is skewed and possibly spread over many years. However, the extent to which observed behaviour translates into actual patterns of parentage is not known. The identification of highly successful males would also allow important mating sites to be identified and, thus, protected in this endangered bat species.

Table 1. *Summary of parentage analysis*

(For the maternity and paternity assignments, candidate parents were selected from a total of 46 sampled females and 28 sampled adult males, in addition to surviving offspring from earlier cohorts.)

year	young born			maternity assignment			paternity assignment		
	cohort size	attached mother	not extracted	unattached	assigned mother (95%)	not resolved	tested	assigned (80%)	assigned (95%)
1993	24	15	0	9	7	2	22	14	5
1994	25	21	0	4	3	1	24	11	4
1995	28	19	0	9	8	1	27	13	4
1996	32	26	0	6	6	0	32	12	5
1997	30	24	2	6	4	2	26	8	5
total	139	105	2	34	28	6	131	58	23

In this study, we used molecular techniques to address the following questions relating to parentage and breeding behaviour in *R. ferrumequinum*.

(i) Is breeding polygynous?
(ii) If male reproductive success is skewed, then does the variance increase or decrease with time?
(iii) Do bats breed within their natal colony?
(iv) Do females preferentially breed with males which are less related to themselves than the colony average?

To determine paternity, we used a likelihood-based method, which has previously been shown to be more accurate than approaches based on exclusion (Marshall *et al*. 1998).

2. METHODS

(a) *Study site and background*

This study focused on a maternity colony in the attic of Woodchester Mansion (near Stroud, Gloucestershire, UK). Bats have been ringed at this site since 1959 (see Ransome 1989, 1990). Since 1982, all juveniles have been caught and ringed soon after birth, most while still attached to their mothers, allowing the identification of mother–infant pairs (Ransome 1990). Adult males have been caught in caves throughout the area during the autumn and winter of each year. This ongoing study has revealed an extreme degree of natal philopatry (Ransome 1995). Annual surveys of autumn and winter sites throughout the area have shown that most Woodchester bats (of both sexes) hibernate locally within several kilometres of the roost, although some individuals disperse further afield, travelling up to 40 km each year in order to hibernate (R. D. Ransome, personal observation). Permanent movements of breeding females between colonies are rare, with just two recorded immigrations in eight years (Ransome 1989).

(b) *Sampling and DNA typing*

During each summer between 1991 and 1997, 3 mm biopsy punches were taken from the tail membrane of all bats present in the breeding colony (Worthington Wilmer & Barratt 1996). The bats sampled comprised mother–young pairs, unattached young, unattached lactating females, non-breeding females and older males. Tissue punches were also collected from males caught at the autumn and winter sites each year. DNA was obtained using a salt–chloroform extraction procedure and all individuals were typed at seven microsatellite loci, as described by Rossiter *et al*. (1999). The sample sizes are given in table 1.

(c) *Parentage analyses*

The program CERVUS was used to determine parentage (Marshall *et al*. 1998). CERVUS calculates the log-likelihood of each candidate parent being the true parent relative to an arbitrary individual and then calculates the difference between the two most likely parents (Δ). Critical values of Δ are determined by computer simulation, which incorporates a realistic rate of sampling error and also removes a proportion of candidate parents to reflect the real world in which not all males are sampled. Critical Δ-values are generated for two scenarios: one where both parents are unknown and one where one parent is known.

The mother–young pairs observed were checked for mismatches. For each cohort, CERVUS was employed to match up unattached offspring and lactating females using a sampling parameter of 100%. Following successful maternity assignment, all unambiguous mother–young pairs were used to infer paternity. For paternity analysis, 28 typed males were considered potential fathers, estimated to represent *ca.* 70% of the total number of candidate males in the population. Based on recapture rates, it is likely that a higher proportion of candidate fathers has been sampled and, therefore, this is a conservative estimate. Like females, males were assumed to be capable of producing offspring in their third year (two years old) and, therefore, surviving males from earlier cohorts were included as potential fathers for offspring born in later years. However, since the birth and death rates were expected to be equal, the input parameters of 28 males and 70% sampling were used to analyse all cohorts. Because females have never been found with close male relatives at mating sites, they were assumed not to breed with fathers or previous male offspring. Therefore, where known, infants' close male relatives were removed from their lists of candidate fathers, since their inclusion can potentially lower the Δ-values and, thus, decrease paternity inference. Although this was not always possible, CERVUS has been shown to be robust to the presence of candidate males which are related to the parents (Marshall *et al*. 1998) and, therefore, their presence is unlikely to affect our results substantially. Based on the few observed mismatches (see § 3), an error rate of 1% was incorporated into the simulation and assignment was carried out at two levels of statistical confidence (80 and 95%).

(d) *Relatedness analyses*

Values of R based on seven loci were calculated for all possible pairwise combinations of the 211 individuals sampled using the program RELATEDNESS 5.03 (see Queller & Goodnight 1989). Pairs of individuals were grouped together and averages calculated to test the expected values of known relatedness classes based on observations and parentage analysis and also to examine the pattern of social structure and breeding behaviour in the colony.

3. RESULTS

(a) *Parentage analyses*

A total of 139 offspring were born over five successive years, of which 105 were found attached to an adult female. Two DNA samples were lost in the extraction process; however, no mismatches were recorded between the remaining 103 putative mother–young pairs (table 1). Out of the 34 offspring that were found unattached, 28 could be matched to a candidate female with 95% confidence. The maternity of the other six offspring could not be resolved, due to a single mismatch (two cases) or identical genotypes of more than one candidate female (four cases).

One hundred and thirty-one unambiguous mother–young pairs were used in order to infer paternity. By comparing the derived values of Δ with the simulation data, paternity could be assigned to 58 young (44.3%) at 80% confidence, including 23 young (17.6%) at 95% confidence. In cases where an offspring could not be assigned paternity with at least 80% confidence, several reasons could contribute to a small Δ-value (see § 4).

(b) *Male reproductive success and breeding behaviour*

In a single year, between 21.4 and 39.3% of the 28 typed candidate males could be identified as successful breeders with 80% confidence. Of those males awarded paternities, their reproductive success ranged from one to three offspring per year (figure 1), revealing a low level of polygyny. However, when the data from all five years were pooled together, 82.1% of the typed candidate males were successful, indicating that the majority of males achieve some reproductive success over a five-year period. In addition, some males were repeatedly successful, with one siring ten offspring, leading to an increase in variance in reproductive success (figure 1).

The identities of males awarded paternities in more than one year, as well as of their female mates, are given in table 2. While no male achieved reproductive success in all five years, three males (8417, 9683 and 9834) achieved success in four years, three in three years and six in two years. Out of the 23 males identified as successful, 19 (82.6%) were born in the Woodchester colony including the most successful individual (9683) and four originated from other colonies. Of the Woodchester males whose birth dates were known, the youngest recorded age of siring was two years old (three examples), while the oldest was 21 years. Table 2 also reveals six cases of where a female bred with the same male in more than one year. Based on the cases where a female bred with two to five identifiable males, we calculated the number of times a parental pair can be expected to reoccur under random

mating. Using a goodness-of-fit test, our observed data and expected values were found to differ significantly, indicating that partner choice is non-random ($\chi_{12}^2 = 42$ and $p < 0.001$).

(c) *Relatedness*

The mean pairwise values of R, which can range from -1.00 to $+1.00$, were calculated for several relatedness classes and social groups and are given in figure 2. As expected, the values between first-order relatives were the highest, with values of 0.46 ± 0.01, 0.44 ± 0.03 and 0.46 ± 0.01 for mother–young, father–young and parent–young, respectively, and 0.51 ± 0.08 for full siblings. The mean R-value for maternal half siblings of unknown paternity (0.28 ± 0.02) differed only slightly from that of known half siblings (0.27 ± 0.03), revealing that full siblings do not occur in high numbers. The mean R-value of average male–female dyads (0.002 ± 0.006) did not differ markedly from that of identified breeding pairs (0.02 ± 0.04), indicating that outbreeding does not occur routinely.

4. DISCUSSION

This study is the first to use molecular techniques to examine the long-term patterns of parentage and male reproductive success in a bat species and provides the most detailed data on bat breeding behaviour to date. Maternity analysis revealed no mismatches between 103 putative mother–young pairs, strongly suggesting that females only suckle their own offspring (Bishop *et al.* 1992) and do not engage in communal nursing (e.g. McCracken 1984). Two cases of a single mismatch occurring between an unattached infant and its most likely mother probably resulted from either a mutation or typing error. Paternity analysis, based on a maximum-likelihood method, revealed a polygynous breeding system, with unexpected patterns of breeding partnership.

Fathers were assigned with high confidence to almost half of the offspring born at Woodchester between 1993 and 1997. We detected a small reproductive skew in each cohort analysed, with a maximum of three offspring sired by an individual male. This indicates that the effective population size is not small, supporting our own findings, of no temporal genetic differentiation between successive Woodchester cohorts (authors' unpublished data). Little or no male reproductive skew has been reported in other temperate bat species (Petri *et al.* 1997; Burland *et al.* 1999), where it has been attributed to aspects of their mating behaviour, which prevent prolonged mate guarding (Burland 1998; Burland *et al.* 1999). In *R. ferrumequinum*, the formation of a vaginal plug, possibly within minutes of insemination (Mann 1964), may contribute to the reproductive skew recorded. However, a larger skew, perhaps expected from high female counts at some mating territories, could be prevented due to the fact that females disperse over a wide area to mate and insemination can continue throughout hibernation, thus preventing males from monopolizing many partners. Moreover, some females appear to eject their plugs prematurely, perhaps allowing further mating (R. D. Ransome, personal observation).
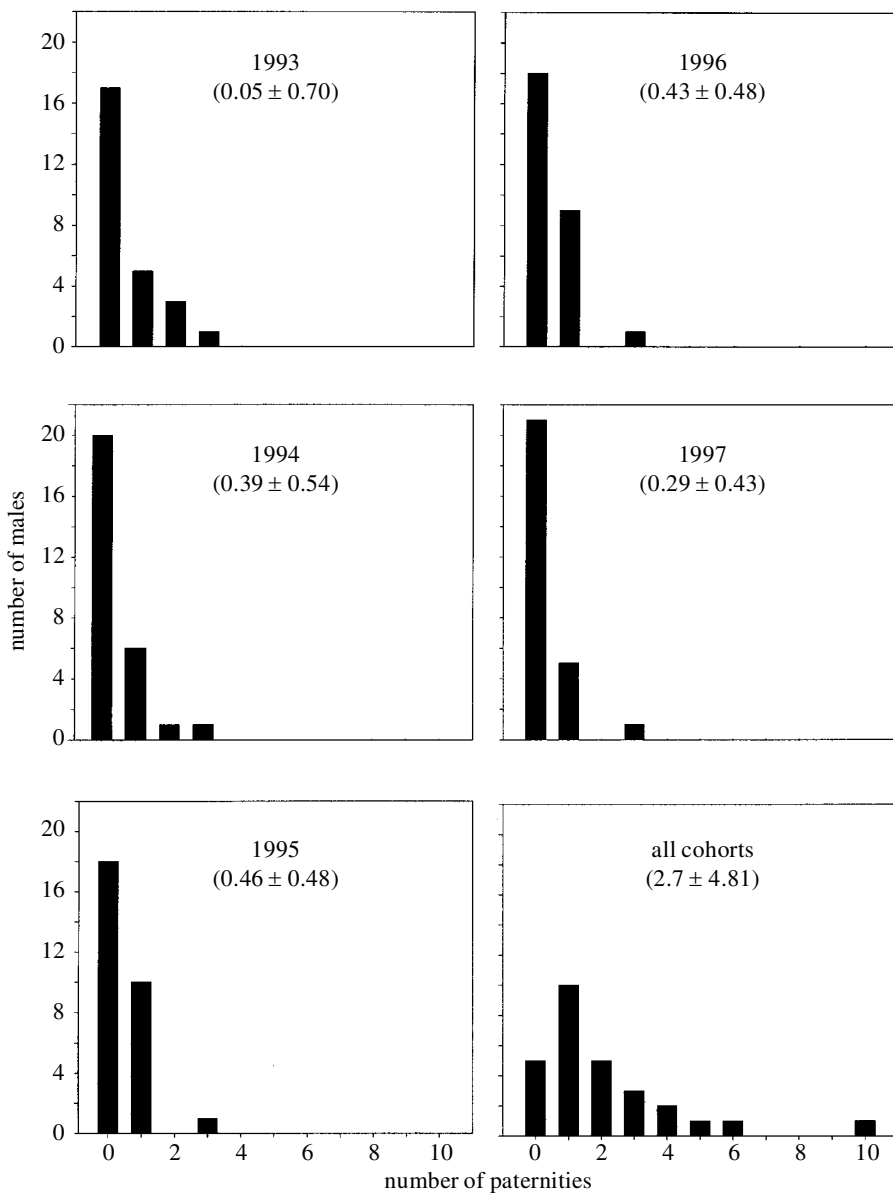
Figure 1. Distributions of paternities awarded at 80% confidence to 23 candidate males for each cohort separately and all cohorts combined. The mean and variance values are given in parentheses.

In comparison, larger male reproductive skews have been found in tropical non-hibernating species, where males defend groups of females for longer time-periods (McCracken & Bradbury 1977; Wilkinson 1985).

Based on the Woodchester-born offspring, the variance in reproductive success increased over five years, due to the repeated success of some bats. We also considered whether the small number of paternities awarded to some males was an artefact of their mortality within the study period. Although this cannot be ruled out in some instances, generally it appeared not to be the case, as many individuals were not awarded paternities in years when they were known to be alive, either by recapture or assignment of reproductive success in later years. Indeed, the variable recapture rates found in some bats, means that male death cannot be inferred confidently from disappearance. Similarly, differences in the sexual maturity of candidate males are also unable to explain the variance, as just seven males were not sexually mature for all five years of the study, of which four achieved considerable reproductive success over the period when they were mature. Since males can father young between the ages of two and at least 21 years, this suggests that the male lifetime breeding success probably varies widely between individuals.

As male bats may achieve paternities in more than one colony, a wider study is needed to confirm that the reproductive success of the Woodchester males remains skewed over a larger scale. Work is also required to identify the separate components of reproductive success and the determining factors which allow some males to attain higher levels of polygyny than others. For example, although females visit males in mating territories, it is unclear whether they choose certain males *per se* or whether they select a specific site and copulate with whichever individual is present. If the former is true, then successful males are presumably characterized by phenotypic traits. However, although too few successful males have been identified to undertake a detailed analysis, the finding that the most successful male (9683) is smaller than average suggests that the phenotypic correlates of success in this species might not be obvious. Alternatively, if females specifically choose breeding sites, then mating is probably based on resource defence, where males

Table 2. *Breeding patterns of males awarded two or more paternities (at 80% confidence)*

(The identities of males and their female partners are given. An asterisk indicates identities signified assignment at 95% confidence, a double asterisk indicates identities which are cases of mate fidelity and a treble asterisk indicates both.)

	females				
	1993	1994	1995	1996	1997
6967	7631	—	—	—	—
	9837	—	—	—	—
7648	7635*	—	—	—	—
	9675	—	—	—	—
	—	8724	—	—	—
8417	9676*	—	—	—	—
	—	—	—	10 201	—
	—	10 034**	—	—	10 034**
9470	8724	—	—	—	—
	9668	—	—	—	—
	8380	—	—	—	—
	—	6677	—	—	—
9471	—	—	—	8379**	8379***
	9664*	—	—	—	—
9673	—	—	10 194	—	—
	—	—	—	10 346	—
9683	8714	—	—	—	—
	—	—	9675**	9675**	9675***
	—	—	6677*	—	—
	—	—	8731*	—	—
	—	—	—	8724*	—
	—	—	—	8156***	8156***
	—	—	—	—	10 361
9834	8379	—	—	—	—
	6677*	—	—	—	—
	—	10 196	—	—	—
	—	9664*	—	—	—
	—	—	—	10 523*	—
	—	—	—	—	10 756
10 030	10 034	—	—	—	—
	—	9675*	—	—	—
	—	8731*	—	—	—
	—	9837	—	—	—
	—	—	8724*	—	—
10 352	—	—	9837***	9837***	9837***
10 541	—	—	—	—	10 753*
	—	—	—	10 535	—
10 562	—	6705	—	—	—
	—	—	5701	—	—
10 939	—	10 017**	10 017**	—	—

compete for good quality territories and reproductive success should correlate with territory tenure. Based on field observations 'resource defence polygyny' has been described in other temperate zone species, including *Pipistrellus pipistrellus* (Gerell & Lundberg 1985) and *Nyctalus noctula* (Sluiter & Van Heerdt 1966) and would be particularly likely in *R. ferrumequinum* if some territories offered particularly good foraging habitat or favourable temperature regimes for torpor. However, whichever is more accurate, our analyses revealed that the mean value of R between breeding couples is no less than the average calculated among male–female pairs and, therefore, unless mating and reproductive success differ widely, females do not seem to outbreed actively. To date, by combining data on highly successful males with

observational data on territory tenure, we have been able to identify several important breeding sites.

Although the relatedness analysis indicated that few full siblings were present, six cases of females breeding with the same male in different years were recorded. Mate fidelity within a polygynous mating system has been described in grey seals, where it may function to increase infant survival by reducing aggressive interactions between adult males (Amos *et al*. 1995). In *R. ferrumequinum*, full siblings could result from sperm competition if some males' sperm are consistently superior or from female fidelity for either males or (where territory occupancy is stable) mating sites. If mate fidelity occurs, we propose two hypotheses to explain the possible adaptive significance of such behaviour in this species.
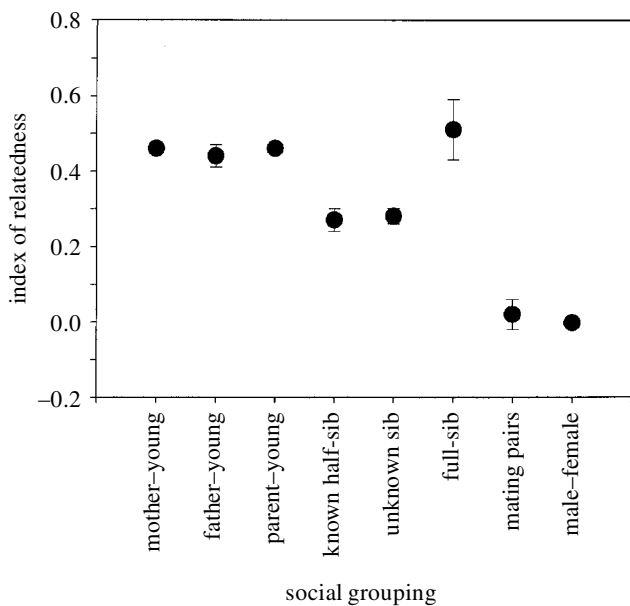
Figure 2. Comparisons of the mean pairwise relatedness values (R) and standard errors calculated between different categories of known and unknown relationships. The values were reached by averaging all pairwise values of relatedness within each group of individuals.

First, females may return to males that they had previously identified as being of good quality, perhaps based on the survival or condition of prior offspring. Second, by mating with the same male more than once, a female will raise the co-ancestry of her offspring. Since *R. ferrumequinum* is highly colonial, the resulting increased relatedness of full siblings may serve to facilitate cooperation among her descendants, thereby increasing their survival and fitness as well as her own. More data will allow these hypotheses to be tested.

The fact that most offspring were not assigned fathers requires further consideration. For confident paternity inference, the difference between the log-likelihood ratios of the two most likely candidate males (Δ) must exceed a critical value. The values of Δ can vary for different reasons (see Marshall *et al.* 1998), for example, due to an assumed low rate of typing error, mismatches between mother–offspring pairs and their most likely fathers gave low Δ-values. Of those offspring not assigned paternity with 80% confidence, a single mismatch with the best candidate male occurred in 27 cases (36.0%) and two or three mismatches occurred in 14 cases (18.7%). In these latter instances, it is probable that the true father has not been typed, for which we suggest three main reasons.

(i) Some females leave the Woodchester area in the autumn and, therefore, may copulate with males born in other maternity colonies. Indeed, ringing studies have recorded a minority of Woodchester females in caves near to neighbouring colonies (D. Priddis and R. D. Ransome, personal observations).

(ii) Females may leave for more transient periods or, conversely, immigrant males may visit the Woodchester area briefly and, thus, escape sampling. Such furtive mating has previously been described in chimpanzees (Gagneux *et al.* 1997).

(iii) It is also possible that a few older Woodchester males remained uncaught as they may have occupied unsampled sites.

Small Δ-values can also result in situations where the second most likely candidate male also closely matches with the offspring. In 23 cases (30.7%) where paternity was not resolved with 80% confidence, no mismatches occurred between an infant and its second most likely father. In such examples, it is more probable that the most likely candidate males were the actual fathers, but were not recognized and, therefore, additional microsatellite markers would improve paternity inference.

Our results reveal that males frequently sire offspring born in their own natal colony. This supports the findings of Burland (1998), who showed that a high proportion of putative fathers originated from within the same colony as their mates, and may contribute to significant levels of genetic differentiation found between Woodchester and its neighbouring colonies (authors' unpublished data). Despite the detected incidence of intracolony fertilization, no heterozygote deficit has been recorded in the Woodchester colony (authors' unpublished data). Therefore, gene flow resulting from some extra-colony copulation, together with occasional migration, is probably sufficient to maintain a considerable level of genetic diversity (authors' unpublished data). Furthermore, the possible presence of several matrilines within the colony (see Wilkinson 1985) may remove the need to disperse in order to avoid breeding with close relatives.

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