Research article

Using behavioural observations and genetic markers to characterise the flexible reproductive system in an ex-situ population of carmine bee-eaters (*Merops nubicus nubicus*)

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Abstract
Combining behavioural observations with population genetic analyses in ex-situ populations can be used to evaluate factors that determine the success of a breeding programme. Avian species living in a colony accrue reproductive benefits by taking advantage of multiple reproductive strategies. Growing evidence suggests that both extra pair fertilisations (EPF) and intra-specific nest parasitism (ISNP) are sufficiently frequent in colonial birds to produce patterns of relatedness that differ from those inferred from behavioural observations. The aim of this study was to use behavioural and genetic information to examine relatedness and reproductive behaviours in an ex-situ colony of Northern carmine bee-eaters (*Merops nubicus nubicus*). We conducted behavioural observations to determine social parents (i.e., the males and females that entered/exited each nest with the highest frequency). We also used microsatellite markers to conduct parentage analysis and calculate relatedness between individuals in the colony. Using two methods of parentage analysis, we were able to determine one or both parents of all offspring. Males and females differed in their parental behaviour during the different reproductive phases. Finally, both males and females were found to use reproductive strategies other than monogamy. In general, EPF and ISNP are two of the consequences of social living, with multiple individuals exhibiting these various strategies for reproduction. In summary, our study found that Northern carmine bee-eaters use a flexible social system that enables individuals within populations to take advantage of suitable nest conditions and mating strategies as they arise, which allows for some individuals in this ex-situ colony to have improved reproductive success.

Introduction

Conducting research on ex-situ avian populations not only provides information that can be applied to improving the daily management of that species, but also presents the opportunity to compare data to natural populations and those species which are difficult to observe in the wild (Bouchard and Anderson 2011; Leighton 2014). Studying reproductive behaviour strategies using behavioural observations combined with population genetic analyses, can help evaluate factors that help determine the success of ex-situ breeding programs. For example, reproductive behaviours, pedigree, and microsatellites have been used to compare effective numbers of breeders in the in-situ and ex-situ populations of bearded vultures (*Gypaetus barbatus*; Guatshi et al. 2003). Effects of inbreeding and levels of mitochondrial diversity were compared to survival and reproduction in the pink pigeon (*Columba mayeri;* Swinnerton et al. 2004) and, in

lesser kestrels (*Falco naumanni*), microsatellites were used to examine individual reproductive success and overall genetic health of individuals produced in ex-situ breeding programmes destined for reintroduction to the wild (Alcaide et al. 2010). Molecular genetic analyses are particularly useful in evaluating the breeding programmes of avian populations of species that live in large flocks or colonies, as well as those that have more complex reproductive systems that are difficult to track using basic pedigree information.

Colonial living in birds has been found to have both positive reproductive benefits as well as costs to both sexes (Rolland et al. 1998; Covas and Griesser 2007). Individuals living in a colony accrue reproductive benefits by taking advantage of the density of individuals and the potential to use multiple reproductive strategies. Pairs may have increased clutch size and higher offspring survival (Stacey and Koenig 1990; Covas et al. 2006). From the perspective of a reproductive female, colonial nesting provides opportunities for intra-specific nest parasitism (ISNP),

or having others incubate and care for her offspring (Emlen and Wrege 1986; Yom-Tov 2001; Covas et al. 2006). Females may have increased production of offspring due to multiple social and/or reproductive male partners through extra-pair copulations (EPC; Li and Brown 2002). Male-specific reproductive benefits include the opportunity for multiple matings through extra-pair fertilisations (EPF) and the avoidance of some parental investment expenses (Covas et al. 2006). Although group living offers some individuals a benefit, this can be at the expense of others (Emlen and Wrege 1986); therefore, there are also costs to colonial living and breeding in high densities. These include uncertain parentage due to EPF, intra-specific cannibalism (Wittenberger and Hunt 1985) infanticide of chicks (Wittenberger and Hunt 1985; Danchin and Wagner 1997) and inter-specific depredation of chicks (Rolland et al. 1998). When ISNP is present, it can lead to reduced fitness in birds raising the chicks of non-relatives.

The family of bee-eaters, *Meropidae*, consists of 26 species with considerable diversity in social and breeding behaviours (Burt 2002; Boland 2004). They may be sedentary or migratory, pair-breeding and/or cooperative, with some species having extremely complex social organisation at nest sites (Burt 2002; Boland 2004). Because of the variation in nesting behaviours, this group has been used to examine questions related to costs and benefits of coloniality, sociality and the presence of various reproductive strategies, such as cooperative breeding (Burt 2002). Wrege and Emlen (1991) studied several colonies of white-fronted bee-eaters (*Merops bullockoides*) to examine ISNP, finding that parasitising females tended to remove host eggs from the nest, and host females frequently discarded eggs that were not their own (Elston et al. 2007). In some species, nests were rarely left unguarded due to threats of predation and ISNP (Burt 2002). In other species of bee-eaters, males are known to guard their mates against EPC, as in the blue-tailed bee-eater (*M. philippinus*; Burt 2002). However, males frequently make use of EPC if provided an opportunity to sire additional offspring without the cost of parental investment (del Hoyo et al. 2001; Elston et al. 2007).

Growing evidence suggests that both EPC and ISNP occur frequently enough in birds to produce patterns of relatedness that differ appreciably from those inferred from observational studies. The presence of these reproductive strategies could have major impacts when examining relatedness and the identities of birds performing behaviours such as ISNP and multiple mating (Jones et al. 1991). Early studies examining relatedness in bee-eaters did not account for EPC or ISNP due to the lack of genetic data. The authors recognised these behaviours were likely occurring, and although assumed to be at low rates, they acknowledged that they may be overestimating relatedness as a result (Emlen and Wrege 1988; Lessells 1990).

Northern carmine bee-eaters (*M. nubicus nubicus*) are native to the savanna woodlands, rivers and grass plains of Africa (Fry 1972). They are opportunistic, insectivorous birds that forage aerially, consuming honeybees, grasshoppers and flying ants (Nickerson 1958; Fry 1984; Fry et al. 1992). While it has been previously determined that carmine bee-eaters are colonial breeders (Nickerson 1958; Fry 1972), little research has been done on the reproductive strategies used in their colonies and very few observational studies have been conducted on wild colonies (Fry 1972). The aim of this study, therefore, was to use genetic and behavioural information to test hypotheses related to life-history parameters, relatedness and reproductive behaviours of individuals in an ex-situ colony of Northern carmine bee-eaters. We hypothesised that alternative reproductive strategies are exhibited by this colonial species and specifically predicted that ISNP and EPF are present. To test our predictions, we used genetic data and behavioural observations. For the birds' behaviour, we predicted that males and females will differ in their parental behaviours throughout breeding season. We determined the family relationships between individuals in an ex-situ colony of Northern carmine bee-eaters using parentage analyses and by comparing relatedness values between individuals. We then used the parentage and relatedness values to interpret reproductive and parental behaviours observed during five breeding seasons. Our analyses allow us to clarify aspects of the behavioural and genetic mating system in an ex-situ colony of the Northern carmine bee-eater. Investigating these factors may improve the understanding of the social and reproductive behaviour of this relatively under-studied species and yield improvements in the management of this, and other, colonial species in ex-situ populations by understanding the complex social structure and balance of a breeding colony.

Materials and methods

Study species
The carmine bee-eater colony examined in this study is held in the behind-the-scenes Avian Research Center (ARC) at Disney's Animal Kingdom, Lake Buena Vista, Florida, USA. The population increased from nine individuals in 2006 to 27 individuals in 2012, through both reproduction and transfer into the colony from other zoological collections (see Elston et al. 2007 for full description of enclosure, diet, and husbandry protocols). In 2005, a new design for the artificial nest box was implemented that gave animal keepers better access to nests and chicks, enhanced hygiene and provided both better airflow and space, in order to promote parental rearing of young (Elston et al. 2007). We increased the number of nest tunnels and nest boxes in the enclosure from five in 2005, to nine in 2006–2010 and to 21 in 2011. Additional husbandry practices were also introduced over time which improved fledging success, including replacing viable eggs with dummy eggs and artificially incubating eggs until they were externally pipped before returning them to the nest boxes, to prevent breakage and potential expulsion from the nest.

Behavioural data collection
Behavioural observations were performed during the excavation, nesting and chick-rearing phases, following the methods in Elston et al. (2007) from April through July in the years 2005–2007, 2009 and 2011. This species' breeding season is compact and highly synchronised, with the entire season from reproduction through fledging lasting less than 10 weeks each year. The colony was observed for 15 minutes in the morning and in the afternoon,
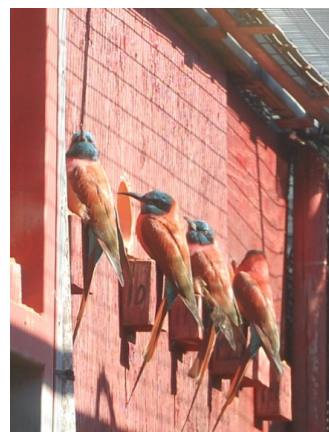

Figure 1. Photo of adult carmine bee-eaters (*Merops nubicus nubicus*) visiting a nest cavity.

approximately five days per week for a total of 113.25 observer hours over five years. Birds were individually identified by coloured bands. All occurrences of birds visiting the nest platform (i.e., landing on the numbered block outside the nest tunnel; see Figure 1), entering and exiting the nest cavity were recorded, along with the identification of each individual. Duration in the nest box was recorded when possible. It was also noted if the individual was carrying a food item into the nest. A single data collector conducted each year of data collection.

Behavioural analyses

Each breeding season was divided into three periods: excavation of nest tunnels, incubation of eggs and rearing of chicks. Nests were considered active during the excavation phase if an adult was observed entering and exiting and if eggs/chicks were present in later phases. The total number of times all individuals visited each nest platform during observations each year was recorded, which may be a behaviour of males who are mate- or nest-guarding, or of birds seeking EPC or ISNP. All comparisons between males and females, and between resident and non-resident males, were performed using mixed models with bird identity and year as random effects, and outcome variables were transformed using the natural log before analysis. To determine which birds were most likely to be guarding nests, the total number of visits per year between males and females, and between resident and non-resident males, were tested for significant differences. To investigate which birds more frequently performed parental behaviours (incubation, brooding and chick provisioning), the total number of times each individual entered or exited a nest cavity each year was recorded. The male and female individuals with the highest frequency of entering and exiting throughout each breeding season were assigned the nest residents and the social parents of the eggs laid in each nest cavity. All other birds that entered or exited at lower frequencies were considered non-residents. Additionally, differences between males and females in average time spent in the nest during the three stages of the breeding season were tested to further examine parental investment. Finally, differences in rates of food provisioning to the nest between male and female parents were tested. All statistics were calculated with JMP Pro 12.1.0 (2015 SAS Institute, Cary, North Carolina, USA).

Genetic data collection

Blood samples were collected in 2009 from 15 bee-eaters during routine physical examinations. Three to four drops of blood (~15 μL per drop) were placed into 1.5 mL tubes containing 1.0 mL of Longmire Buffer (100 mM Tris HCL pH 8.0, 100 mM EDTA, 10 mM NaCl, 0.5% SDS; Longmire et al. 1988). Tissue (liver or muscle) was collected from 32 deceased embryos or chicks between 2009 and 2012 and preserved in ethanol. Contour feathers were also collected opportunistically from 14 juvenile bee-eaters in 2011 and 2012. In total, DNA samples from 61 individual birds (adults, embryos or chicks) were collected. Sampling for this study began in 2010, so prior to this year only living birds were sampled; however, all fertile eggs, chicks and adults were sampled in 2010 and 2011.

Genomic DNA from tissue and blood was extracted using a standard phenol-chloroform extraction protocol (Sambrook and Russell 2001). Genomic DNA from feathers was extracted using a DNeasy Blood & Tissue Kit (Quiagen, Valencia, California, USA) with modifications as described in Bush et al. (2005). The concentration of DNA in each sample was determined with a Nano-Drop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) and was visualised on a 1% agarose gel. All samples were diluted with double-distilled water to a final concentration of 20 ng μL^{-1}.

We generated eight species-specific microsatellite loci and tested an additional six loci originally isolated in *Merops apiaster* which also amplified in *Merops ornatus* (Dasmahapatra et al. 2004; Adcock et al. 2006). We genotyped 61 individuals at these 14 loci (see Supplementary information 1, for microsatellite loci generation, PCR conditions and genotyping methods).

Genetic analyses

Eight of the 14 loci were found to be polymorphic and were therefore used for further analyses (Table 1). The software program FSTAT2.9.3 (Goudet 1995) was used to test for deviation from Hardy-Weinberg Equilibrium and for evidence of linkage disequilibrium. The program Micro-checker (van Oosterhaut et al. 2004) was used to check for the presence of null alleles and scoring errors, such as peak stuttering or allelic dropout. The probability of identity (PI) of the eight polymorphic loci was also estimated, using the programme Gimlet (v1.33; Valière 2002). Gimlet was also used to calculate the observed and expected heterozygosities for the microsatellite loci used in this population.

From 2006 to 2011, 14 potential sires and 12 potential dams were present in the colony. A bird was considered a potential parent if it was present when eggs were laid (May and June of each year). Eleven candidate sires and 10 candidate dams were genetically sampled, leading to a probability of 78.6% and 83.3% that the sire and dam were included in the sampled dataset, respectively. These a priori probabilities were used in both CERVUS (v3.0.3; Kalinowski et al. 2007) and COLONY (v2.0; Jones and Wang 2009) for parentage screening (see Supplementary information 1, for program settings). The remaining potential parents (3 potential sires and 2 potential dams) were not sampled because they died before our samples were collected. During this time period, genetic samples were collected from a total of 50 offspring (chicks or fertile eggs/embryos). By examining hatching dates and each potential parent's timeline of presence in the colony, it was possible to exclude some candidate males from paternity analysis for 29 of the offspring, and some candidate females from maternity analysis for all 50 offspring with varying numbers of potential sires and dams. In both programmes, parentage is assigned with confidence levels of 95% (strict) and 80% (relaxed) confidence. Both programmes (CERVUS and COLONY) were used for parentage screening because they use different methods to assign parents, and are known to differ in levels of confidence in assignment, with COLONY typically producing more assignments at a higher confidence level (Ferrie et al. 2013). A visual comparison between the genotypes of the offspring and those individuals identified as their social parents was also performed, and the number of loci mismatched between the offspring and their social parents was determined. Finally, to further examine the relationships between all individuals in the colony, and to determine relationships that were not assigned in parentage analyses, relatedness was estimated between all individuals using Coancenstry (Wang 2011). All three programmes assume a genotyping error rate of 0.02 as suggested by Wang (2004).

Application of genetic analyses to investigate behaviour

Life history parameters, and occurrence of various reproductive strategies for this species, including age at first and last reproduction, clutch size, multiple paternity in clutches, ISNP and males with multiple mates, were examined. In these analyses, only genetic parentage assignments that were assigned with >95% confidence in both programmes, unless specifically noted, were considered. To investigate if inbreeding is occurring, the relatedness of behavioural parents at each nest was examined and, when both parents of an egg were assigned with >95% confidence in both COLONY and CERVUS, relatedness of the genetic pair was examined.

Locus	Primer Sequence (5'-3')	Ta (°C)	Repeat motif	Size (bp)	Number of Alleles	Allelic Richness	H_{exp}	H_{obs}	$PI_{unbiased}$	PI_{SIB}	NE-PP
Be19.2‡	*GTCAAGTGGGCTGTTG GAGAAGAGGGGCTACTT CCAAGC	59	(AC)9	189-211	9	8.796	0.82	0.82	0.04408	0.3517	0.16
Be2.31‡	*CTTCAGGCAAGTGACC ACAGCAGAGGGACACC AGAGCTTC	61	(AC)13	183-211	11	10.719	0.79	0.61	0.002470	0.1315	0.18
Mnub103	*TGTGATGCAAAAAGCC AAGAGGCAGGCTGGCT GGTTTTGTCC	62	(GATA)5(GACA)¬¬3	231-263	6	5.965	0.70	0.64	0.0003068	0.05731	0.13
Mnub102	*GAATGATATGTCATGG GGGAATCAAAAAGGTT CGCCATCACT	62	(GATA)8	163-179	5	4.783	0.58	0.59	0.00006349	0.01500	0.47
Mnub107	*CATCAGCCCATTCACA AAAGACTGCATCCAGCT TGCAGAACACGA	51	(CTAT)5	231-247	5	4.887	0.58	0.46	0.00001394	0.007761	0.44
Be2.46‡	*AATGGCTGTAAGTGG TCATGGTGATTTCATCC CAGATGTGC	59	(AC)5n3(AC)3n8(AC)8	198-207	4	4.000	0.55	0.59	0.000003120	0.002197	0.49
Mnub105	*ACACATTGCCATGAG GACAGCTGCACATGG ATGCTTTTTGC	60	¬+(GATA)¬¬12	237-269	7	6.726	0.61	0.80	0.0000007052	0.02899	0.49
Mnub104	*ACATTGCCATGGGGA CAGCTGATTTGAATGC TGCTGTTTGC	60	(GATA)13	190-222	7	7.000	0.57	0.61	0.0000001874	0.004124	0.48
Mean							0.65	0.64			

Table 1. Characterisation of eight microsatellite loci used in carmine bee-eater (*Merops nubicus nubicus*) identity and parentage analysis. Loci were amplified using PCR conditions described in the text. Three loci (identified by the symbol ‡) were originally identified in Dasmahapatra et al. (2004). Loci are sorted by rank of cumulative unbiased probability of identity (PI), which was calculated using Gimlet v1.33 (Valière 2002). Number of alleles and allelic richness (based on minimum sample size of 47 diploid individuals) were calculated with FSTAT 2.9.3 (Goudet 1995). Expected and observed heterozygosity were calculated in Gimlet v1.33. The asterisk (*) in the primer sequence denotes a 5' tail (TGTAAAACGACGGCCAGT) attached to the primer sequence; + in the repeat motif represents an imperfect repeat in the sequence; Ta, annealing temperature; H¬exp, expected heterozygosity; Hobs, observed heterozygosity; PIunbiased, unbiased probability of identity; PISIB, probability of identity in a population of all siblings. Note that values for PIunbiased and PISIB are cumulative, such that the value listed for Mnub104 is the cumulative effect of all loci. NE-PP is the non-exclusion probability of the parent pair from CERVUS (Kalinowski et al. 2007) results.

Results

Behavioural analyses

In five years of observations, 30 of the 50 offspring's social parents (individual male and female) were determined through observations of birds entering/exiting the nests (Figure 2; Table 2 in Supplementary information). The social parents were considered the resident of the nest tunnel, and all birds that visited at lower rates were considered non-residents of that nest tunnel. Throughout all stages of the breeding season (excavation, incubation, chick rearing), birds other than the social parents were also observed visiting the nest tunnel. Males were observed visiting the nest tunnels more often than females, although this was not significant (F=3.14, DFDen=26.4 p=0.09) and there was no evidence that social parent or resident males visited more than non-resident males (F=0.54, DFDen=37.0, p=0.47).

Prior to chicks hatching, multiple birds in addition to the social parents were observed entering and exiting nest tunnels. Among all active nest tunnels and prior to chick hatching, 66.67% were entered/exited by non-social parents during excavation and incubation. When examining differences during the three periods of the breeding season and between the two sexes, the duration of time spent in the nest tunnel was significantly different between sex (F=16.82, DFDen=19.4, p<0.001) and period (F=12.88, DFDen=104.0, p<0.0001) with females spending longer in the nest than males, and incubation period having the longest stay time by females (Figure 3).

When examining the rate of food provisioning (number of food items brought to cavity per hour) by each parent, there was no difference between males and females (F=0.61, DFDen=25.1, p=0.44). Food provisioning by non-social parents was only observed twice throughout the study.

Genetic analyses

The eight polymorphic loci were found to be in Hardy-Weinberg equilibrium using the Bonferroni corrected significance value of α=0.00625, and there was no evidence of linkage disequilibrium between loci based on the Bonferroni corrected significance value of α=0.001786. Locus Be2.31 showed signs of null alleles when analysed with Micro-checker; however, there was no evidence of scoring error due to stuttering or of allelic dropout. The cumulative unbiased probability of identity (PI; over all loci) was 1.874×10^{-7}, suggesting that approximately one in 10 million genotypes will match by chance alone and the PI in a population comprised of full siblings was 2.197×10^{-3}, suggesting that approximately one in 450 genotypes would match by chance if all individuals were full siblings. Therefore, these eight loci show sufficient discrimination ability and were used in parentage screening and relatedness analyses.

Figure 2. Total enters and exits of each nest tunnel per individual bird by year. The birds that had the highest number of enters/exits combined per year were considered the social parent of the eggs/chicks in the nest. Grey highlighted nest tunnels are those that had eggs/chicks present. The male and female with the most enters/exits to each nest are identified by ♂ and ♀ symbols.
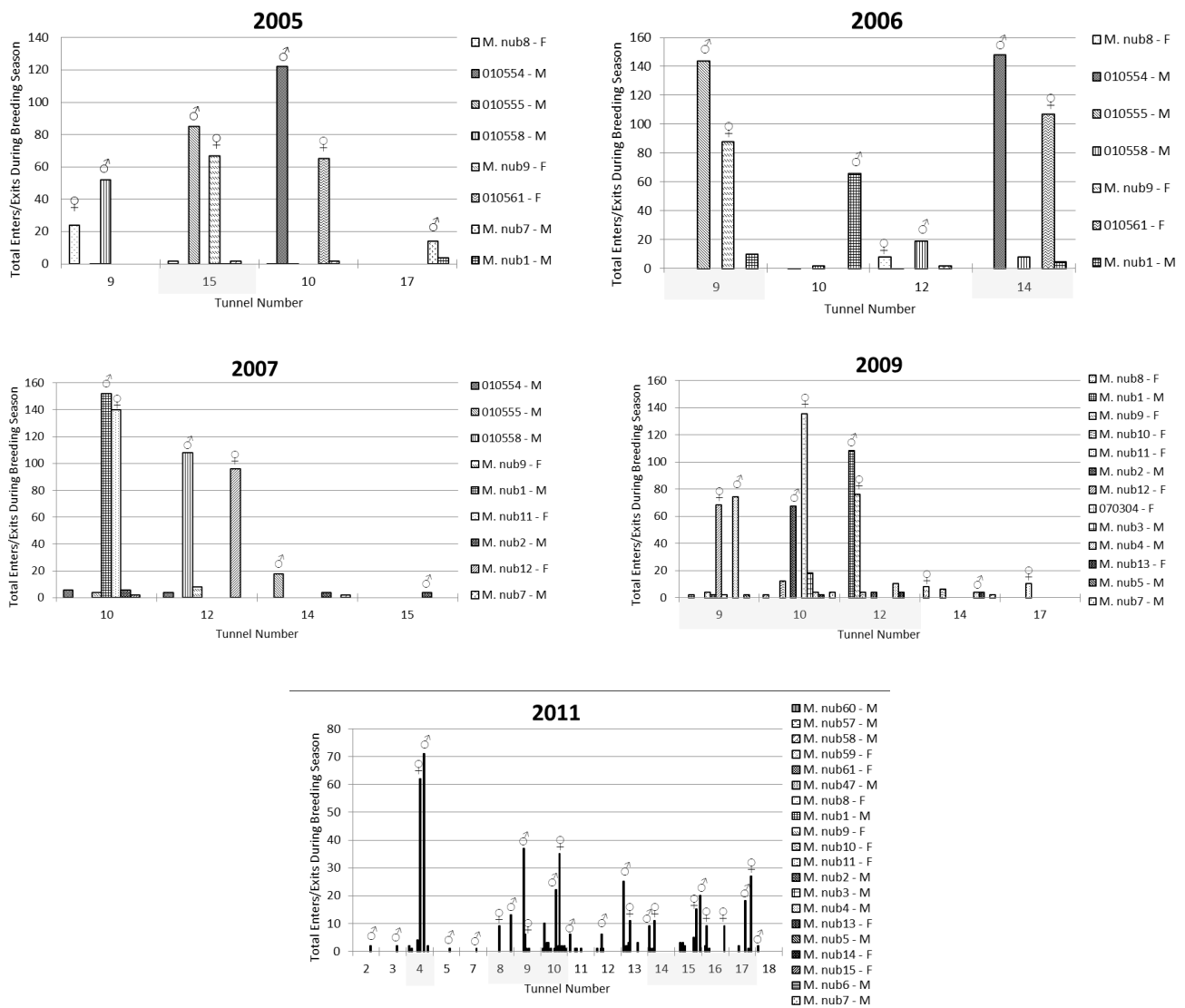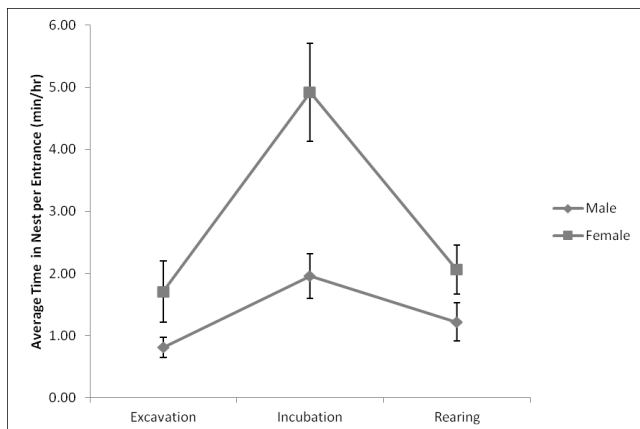

The parentage of each offspring hatched in the population, obtained from the behavioural observations and the two methods of assignment based on molecular markers (COLONY and CERVUS), are shown in Table 2 in Supplementary information. We analysed parentage in 50 offspring (embryos and chicks) and thus there were 50 potential pairings. Of the two assignment programmes, analyses from COLONY resulted in more sire and dam assignments with higher confidence (sires: 35 with strict, 4 with relaxed, 7 with low confidence and 4 not assigned a sire; dams: 31 with strict, 3 with relaxed, 13 with low confidence, and 2 not assigned a dam) than the assignments from CERVUS (sires: 20 with strict, 16 with relaxed, 13 with low confidence, and 1 not assigned a sire; dams: 19 with strict, 20 with relaxed, 10 with low confidence, and 1 not assigned a dam; Table 2). In comparing the congruence of assignments between the two programmes, 34 assignments of sires were the same in both programs, and 35 assignments of dams were the same. Non-matching assignments are also displayed in Table 2.

Application of genetic analyses to investigate behaviour

Basic life-history parameters for this species were determined from the results of parentage analyses. Both males and females were reproductively viable before they reached one year old (eggs are laid or fertilised at slightly older than 11 months). The oldest male to have fertilised an egg was at least 17 years old, and the oldest female to lay a fertile egg was at least 13 years old. Both birds were wild caught and have estimated hatch years, so these ages may be inaccurate. One female was assigned in 2010 as the dam to four eggs with >95% confidence and an additional three eggs with >80% confidence, suggesting female fecundity may be 1–7 eggs per breeding season. These eggs were spread out across three nests, such that total fecundity is larger than clutch size, which was observed to be 1–5 eggs per nest with an average of 2.8±1.08 in 2010 and 2011 (years for which complete nests were sampled, see Table 2 in Supplementary information). The observed clutch size is slightly greater than previous estimates by Nickerson (1958), who determined that *M. n. nubicus* had clutch sizes ranging from 1–3 in natural colonies.

Figure 3. Average time in nest per entry/exit (min hr⁻¹) of all males and females during three nesting phases: excavation (M=10, F=10), incubation (M=13, F=11), and [chick] rearing (M=10, F=10). Mean±SE for each phase.

both social and genetic pairings of parent to offspring, between full or half siblings and grand-parent to grand-offspring. Only two of these genetic pairings (one father–daughter, one half-sibling) resulted in living, inbred offspring.

After chicks began hatching, individuals that were not the social parents entered or exited 24.24% of tunnels. The total time non-social parent individuals were observed in the tunnels was low (98.12±48.79 sec) across the four weeks post hatching. In just two cases, a non-social parent bird (in both cases a male) was observed entering with food. It was not possible to assess the relationship of the first bird to the parents of this nest, as the individual was not included in our genetic sampling. The second individual was likely to be unrelated to the male parent of the nest (r=0.07), but was identified as the offspring of the female parent of this nest-box location from the previous year (r=0.62, >95% confidence dam assignment in both COLONY and CERVUS).

Discussion

In this study, behaviours related to parental investment and reproductive output were observed during the breeding season, and genetic analyses were used to determine parentage and relatedness between individual Northern carmine bee-eaters in an ex-situ colony. Behavioural differences in male and female parents were examined, revealing differing levels of contribution to behaviours by each sex depending on the phase of the breeding season. Using two methods of parentage analysis, it was possible to determine one or both parents of all 50 offspring from the colony with at least 80% confidence. Parentage assignments were supplemented by performing a visual comparison of the number of loci mismatching between social parents and genetic assignments, as well as calculating relatedness values between all individuals. Finally, it was found that birds made use of alternative reproductive strategies including ISNP and EPF.

Although not significant, males were observed visiting the nests more than females, reflecting a behaviour that may play a role in reproduction and levels of parental investment. At the beginning of the breeding season in wild colonies of carmine bee-eaters, visiting nests, but not entering, may serve as a reproductive strategy for either resident or non-resident males (Fry 1972). For the former, it may be advantageous to guard exiting females or nest contents. For the latter, nest visiting can provide EPF or nest-guarding opportunities (Burt 2002). In our study, females were observed spending more time than males in the nest during the incubation period (Figure 3) suggesting that females invest time incubating and tending to eggs, whereas males may play a greater role in guarding the nest against intra- and inter-specific predation. Indeed, males were often observed perched nearby the holding box during the incubation period (Elston et al. 2007). However, the present study found no difference in duration spent in the nest between males and females during excavation and chick rearing (Elston et al. 2007; Figure 3). Females may reduce their maternal investment during these phases due to the high energy requirements of laying and incubating eggs, and as a measure against ISNP (Emlen and Wrege 1986), or they may be biologically equipped to incubate eggs as obvious brood patches have been described in the females of black-headed bee-eaters (*M. breweri;* Schmidt and Branch 2005). More research into sex differences in parental care and investment may uncover why these differences exist.

When observing rates of food provisioning to chicks, there was no difference between male and females, also supported by the findings of Elston et al. (2007), and suggesting that both parents provide equal investment during rearing, and that males are either confident in their paternity or cannot distinguish between chicks that are not their genetic offspring. In fact, as a husbandry

When comparing the social parents to the genetic parents, 15 sires and 15 dams were both socially and genetically identified as parents, with both assignment programmes giving the same parentage assignment, although the confidence ranged from no confidence to high confidence in the assignments (Table 2). In all cases where both programmes assigned the same individual as the social parent, the visual comparison of genotypes matched completely, with the exception of four cases in which one loci mismatched. Also, an additional six sires and four dams matched as social parent and genetic parent in one of the assignment programmes, where two loci mismatches were identified. However, four sires and five dams whose genetic assignments matched in both programmes, but the social parent assignment did not match, suggesting the presence of EPF and/or ISNP. The number of loci mismatched in these cases ranged from 0–7. Clutches with multiple paternity were found in both 2010 and 2011 (Table 2). In 2010, tunnel 17 had two eggs laid by the same female that were sired by different males (>95% sire assignments COLONY; Table 2). Females were also found to parasitise the nests of other pairs; specifically, four instances of ISNP were found in 2011 (>95% dam assignments COLONY; Table 2). Males were found to mate multiply and use a strategy of EPF. In 2011, parentage assignments of males that had offspring with their social mate, but also sired offspring with other females, was confirmed with >95% confidence in two cases. In these cases, where ISNP or EPF are probable, there are almost always mismatches in the genotypes between offspring and social parents (Table 2). In total, when both methods assigned parentage with high confidence, 28.6% of all nests were found to have ISNP by females (4 of 14) and 14.3% of nests to have EPF by males (2 of 14) across the four years when both genetic and behavioural data were collected.

The average relatedness in the colony was 0.11±0.004. Social pairs were related to each other with an average relatedness of 0.14±0.05, and genetic pairs that were assigned to an offspring with >80% confidence had an average relatedness of 0.09±0.05. Comparing the parentage results and relatedness values, it was observed that most pairs, both social and genetic, were unrelated (11 of 16 and 10 of 12, respectively). However, it was also observed

practice in this colony to reduce egg breakage, eggs are incubated, and then not necessarily returned to the tunnel in which they were laid. While this may constitute a confounding factor in studying parental behaviour, we do not have any evidence to suggest that the birds provide less care to chicks that are not their own; indeed, the rate of food provisioning to chicks was similar across nest boxes (Elston et al. 2007, G. M. Ferrie personal observation) and males of many avian species do not have the ability to discriminate kin (Kempenaers and Sheldon 1996). These results suggest that Northern carmine bee-eaters invest equally in feeding offspring regardless of parentage; future studies that specifically manipulate which nests chicks are returned to could test kin recognition (Komdeur and Hatchwell 1999) and thus parental investment in this ex-situ environment.

Regarding cooperative breeding in this species, it is not possible to confirm whether or not helpers are present during chick rearing. Provisioning by non-parental birds was only observed twice in five years: in 2007 we observed an unpaired adult male enter one nest with food three times, and in 2011 we observed a juvenile male, who was paired and had successfully fledged chicks at a nearby nest, provision the nest that was located ~0.5 m directly below his own nest three times. It was not possible to assess the relationship of the first bird to the parents of the provisioned nest and the second individual was related only to the female parent of the provisioned nest. This one observation of possible helping at a related female's nest may have been a random occurrence. While males are more commonly observed as helpers in bee-eaters (Brooke 1994) and that it is common to have grown offspring remaining to assist in rearing young (Emlen and Wrege 1992), more regular observation of provisioning by non-parents will be necessary to confirm that helpers are present at the chick-rearing stage of the breeding season in this ex-situ colony (Boland 2004). The study may not have detected typical helping behaviour in this colony for the following reasons. First, food resources are provided to the birds, and while not unlimited, there is not much food competition, thus more birds can dedicate energy to reproductive attempts at their own nest rather than to foraging. Nest sites are also not limited, as every year some nests are not used. Second, the average relatedness in the colony was ~0.11, or at a level less than half-siblings. Perhaps colony relatedness needs to be higher before the benefits of helping kin outweigh the costs of attempting reproduction. Finally, it is also possible that Northern carmine bee-eaters are not cooperative, as in their sister taxon (Emlen 1990).

As predicted, both males and females were found to use reproductive strategies other than monogamy. From the female's perspective, clutches with multiple paternity were observed and females were also found to parasitise the nest of other pairs. Both fecundity and clutch size were larger than observed in wild populations, which may be an artefact of the resource-rich ex-situ environment enhancing levels of ISNP and leading to larger clutches. Males were found to mate multiply and use a strategy of EPF. EPF may occur more frequently in this population, as only cases of high confidence assignment which were supplemented with visual comparisons of number of mismatches of loci in genotypes (Table 2 in Supplementary information) were considered. These behaviours are all common methods that both males and females use to increase their reproductive output and reduce parental care. Paired males make use of a mixed mating strategy in which the male, while remaining monogamously paired, takes advantage of extra pair opportunities, made possible by synchronised colonial breeding (Trivers 1972; Emlen and Wrege 1986; Rohwer and Freeman 1989). White-fronted bee-eater females were found to parasitise the nests of parents or close relatives, suggesting that some hosts will tolerate ISNP by close kin (Emlen and Wrege 1986). However, white-fronted bee-eaters will toss out eggs that

have been dumped in their nest by parasitising females and are highly territorial, ejecting intruders from their nests with physical contact (Emlen and Wrege 1986; Boland 2004). In this population of Northern carmine bee-eaters, eggs were observed being thrown out of nests and breakage was observed within the nest, which may indicate females removing eggs that are not her own. Long lasting aggressive interactions were also observed between individuals in the nest tunnels. ISNP may pose the primary threat to certainty of parentage in bee-eaters, even more so than forced copulations resulting in EPF (Emlen and Wrege 1986). Future studies should compare the rates of these behaviours to determine their frequency.

In general, forced or voluntary EPC and ISNP are two of the consequences and costs of social living (Emlen and Wrege 1986; Yom-Tov 2001), providing fitness benefits for some individuals, but not all. Birds remain in colonies and sustain these potential costs, suggesting that the selective advantage of colonial living outweighs the costs (Emlen and Wrege 1986). In some species, breeding pairs exhibit moderately high levels of social monogamy, with pairs of European and white-fronted bee-eaters re-nesting together at 88% and 87% of nests, respectively (Lessells and Krebs 1989; Emlen 1990), and both sedentary and migratory bee-eaters exhibit similar rates of pair fidelity (Boland 2004). However, in the study colony, some pairings occurred with the same mate in subsequent seasons (3 pairs in 2004 and 2005, as in Elston et al. 2007), whereas others paired with a different individual year after year. With more years of observation of a greater number of pairs, it should be possible to obtain a better understanding of how often pairs choose the same mate the following year, and how often they find a new mate. However, the re-pairing rate in the study colony may not be comparable to large, migratory, natural populations, as our population is relatively small and does not have a natural ability to migrate or disperse. In bee-eater species, inbreeding is avoided by using a strategy in which females disperse to join new colonies and the social organisation comprises patrilocal extended family groups or clans (Emlen and Wrege 1992). At this time, the study colony is not managed this way, as there is no emigration from the colony; however, there is occasional immigration in the form of newly-introduced birds. There have been examples of inbreeding in the colony and this may be underestimated, as the acceptance of parentage assignments only with >80% probability is conservative. Future colony management should aim to include a strategy that allows for dispersal of females to avoid inbreeding when choosing a mate.

In summary, the present study found that bee-eaters in an ex-situ setting use a flexible social system that enables individuals to take advantage of suitable nest conditions and mating strategies as they arise, including EPF and ISNP (Boland 2004). These strategies allow the birds to improve individual reproductive output while taking advantage of the high density of individuals and nest sites and the greater foraging efficiency which are characteristic of colonial living (Beauchamp 1999). Future studies or experiments could examine these reproductive behaviours to determine if individuals maintain one or multiple strategies, or if they choose different strategies each year. Also, as colony size increases and resources such as nest tunnels or living space become more limited, birds may become more likely to choose a strategy based on their age or experience or some other factor. The study colony has grown from nine to 27 individuals and the number of nest tunnels provided was increased from five to 21. While nest tunnels have not yet been limiting (the maximum number excavated in 2011 was 16 and only 8 of these tunnels were used by a pair throughout the entire season), this is obviously a finite resource which has the potential to influence pair success, mate choice and other factors in reproduction (Yom-Tov 2001). This study provides some insight into the various

reproductive strategies present in an ex-situ colony of Northern carmine bee-eaters. Due to the small size of the colony compared to natural settings, the frequency of these behaviours may not be extrapolatable to an in-situ colony; however, little information currently exists from observational field studies on this species. Therefore, this study may serve as a comparative baseline for future studies on the reproductive behaviour of this species in their natural environment. Furthermore, the study offers a better understanding of the complexity of colonial managed species, which can be of use to zoological managers concerned with reproduction of birds in an ex-situ setting. The methods can be used to evaluate management consequences including effective number of breeders and the impact on loss of genetic variability (Gautshi et al. 2003) in a colonial setting.

Acknowledgements

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