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SUBORDINATE MALES SIRE OFFSPRING IN MADAGASCAR FISH-EAGLE (*HALIAEETUS VOCIFEROIDES*) POLYANDROUS BREEDING GROUPS

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ABSTRACT.—The island endemic Madagascar Fish-Eagle (*Haliaeetus vociferoides*) is one of the most endangered birds of prey. Certain populations in west-central Madagascar sometimes exhibit a third, and sometimes a fourth, adult involved in breeding activities at a nest. We applied DNA fingerprinting to assess relatedness among 17 individuals at four nests. In all nests with young, a subordinate rather than the dominant male sired the offspring. Within-nest relatedness comparisons showed that some dominant males had an apparent first-order relationship with the female. Between-nest relatedness comparisons showed that some adults had an apparent first-order relative at another nest in the study area. Findings that subordinate males contribute to breeding, and that adults in an area may be related, may require conservation measures such as translocation to assure the species' survival.

KEY WORDS: *Madagascar Fish-Eagle*, Haliaeetus vociferoides; *DNA fingerprinting, mating system, nest helper, polyandry.*

MACHOS SUBORDINADOS ENGENDRAN DESCENDENCIA EN GRUPOS DE REPRODUCCIÓN POLIÁNDRICA EN AGUILAS PESCADORAS DE MADAGASCAR (*HALIAEETUS VOCIFEROIDES*)

RESUMEN.—El águila pescadora endémica de la isla de Madagascar (*Haliaeetus vociferoides*) es una de las aves rapaces más amenazadas de extinción. Algunas poblaciones en el occidente-centro de Madagascar exhiben algunas veces un tercero y a veces un cuarto adulto involucrado en las actividades reproductivas en un solo nido. Aplicamos un análisis de ADN para evaluar el parentesco entre 17 individuos de cuatro nidos. En todos los nidos con juveniles, un macho subordinado mas que el dominante engendro la prole. Las comparaciones de parentesco dentro de los nidos mostró que algunos machos dominantes tenían aparentemente una relación de primer orden con la hembra. Las comparaciones entre nidos mostraron que algunos adultos tuvieron un pariente de primer orden en otro nido dentro del área de estudio. El hallazgo de que los machos subordinados contribuyen a la reproducción, y que los adultos en un área pueden estar relacionados entre si, pueden hacer necesarias medidas de conservación tales como traslados para asegurar la supervivencia de la especie.

[Traducción de César Márquez]

The island endemic Madagascar Fish-Eagle (*Haliaeetus vociferoides*) is considered critically endangered (Collar et al. 1994). With 63 known breeding pairs, and an estimated total breeding population of 100–120 pairs (Rabarisoa et al. 1997), it is among the most endangered birds of prey in the world (Langrand and Meyburg 1989, Watson et al. 1993, 1996). Madagascar Fish-Eagles exhibit an unusual dispersal and breeding strategy, possibly restricting the species' distribution and abundance

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through limited dispersal or occurrence of inbreeding. Breeding was believed to be monogamous, but at 46% of known nests, a third, and sometimes a fourth adult is involved with the breeding activities of the primary pair (Watson et al. 1999). Based on banding studies at several nests (Watson et al. 1999), extra-pair birds were believed to be progeny (possibly only male) from previous years. Such delayed dispersal can result in formation of cooperative breeding groups, a relatively rare breeding system among birds (Stacey and Koenig 1990, Ligon 1999), especially among raptors (Simmons 2000, and references therein). Ecological or behavioral factors may influence evolution of cooperative breeding strategies (Newton 1979, Oring 1986, Faaborg and Bednarz 1990, Stacey and Koenig 1990, Sherman 1995), and contribute to attendance of additional adults at Madagascar Fish-Eagle nests. Understanding dispersal and reproductive strategies is critical for developing a management plan to ensure the species' survival.

DNA markers have been applied to a variety of questions regarding conservation of birds (Haig and Avise 1996). DNA fingerprinting proved useful to assess relatedness at the nest (Westneat 1990, Wetton et al. 1992, Haig et al. 1993, 1994a, 1994b) and population (Triggs et al. 1992, Fleischer et al. 1994) levels, to infer species-level population genetic structure (Longmire et al. 1991), and to estimate relatedness in captive stocks (Kirby 1990: 239). We used DNA fingerprinting to determine paternity among Madagascar Fish-Eagle adults attending a nest, and to examine the level of relatedness among adults within and between nests.

METHODS

Samples. We studied three trios and one quartet of fish-eagles at a site in west-central Madagascar (19°S, 44°30′E) on a daily basis during one breeding season from 24 June–5 October 1999. The area is tropical deciduous dry forest containing several lakes (3.09–4.86 km^2) and supports 11 fish-eagle territories (Rabarisoa et al. 1997). Eagles were marked and are referred to by number. Nest sites are referred to by location and nest number (Ankerika 4, Befotaka 2, Befotaka 3, and Soamalipo 2). A dominance hierarchy was observed at each nest based on aggressive interactions between adults. Aerial pursuits (chasing) and physical displacements from either the nest or from perches within 200 m of the nest tree, often accompanied by a distinctive 'displacement' call, were observed throughout the breeding period and were interpreted as signs of aggression (Tingay 2000). Males are referred to as either dominant (α), or subordinate (β or γ). We were unable to establish the dominance hierarchy at nest site Befotaka 3. Nestlings were briefly removed from the nest at ca. 7 wk of age and banded. Blood (0.1–0.25 ml) was taken from the brachial vein (Tingay 2000), immediately placed in 4.5 ml of lysis buffer (100 mM, pH 8.0, 100 mM EDTA, 10 mM NaCl, 0.5% SDS) in a polypropylene tube, labeled, and stored at ambient temperature.

DNA Purification. Approximately 200 μl of blood/buffer solution was placed in 800 μl lysis buffer for 10 min. Protein digestion was performed with 500 μl of supernatant from the first step, 500 μl of fresh lysis buffer, and 0.5 mg/ml proteinase K, with incubation at 37°C overnight. Extractions were performed in 1:1 phenol: chloroform, and 24:1 chloroform: isoamyl alcohol. DNA was precipitated using cold 95% ethanol and 5% sample volume of 5M (0.082M final) ammonium acetate. DNA was resuspended in 25 μl deionized water and stored at −20°C.

DNA Fingerprinting. DNA samples were digested separately with *Hinf*I, *Rsa*I, and *Hae*III. Digests were loaded onto 1% TBE agarose gels (20 cm × 24 cm), and subjected to electrophoresis (Sambrook et al. 1989) at 32 V for 25 hr. Identity Sizing Standard (Lifecodes Corporation, Stamford, CT) was placed in several lanes of the gel to provide molecular weight markers. DNA in the gel was stained using ethidium bromide, photographed using UV luminescence, and transferred (Southern 1975) onto a MagnaCharge 0.45 micron nylon membrane (Micron Separations Inc., Westborough, MA). Jeffreys et al. (1985) and Jeffreys (1987) minisatellite probe 33.15 was hybridized using the NICE hybridization solution (Lifecodes Corporation, Stamford, CT) onto digested, immobilized DNA. Both the 33.15 probe and Identity Sizing Standard were labeled with NICE chemiluminescence. Unhybridized probe and size standard were washed from the membrane using Quick-Light wash solutions (Lifecodes Corporation). The hybridized probe was illuminated with Lumi-Phos 480 (Lifecodes Corporation) and visualized by exposure to Kodak XAR5 X-omat film.

DNA Fingerprinting Analysis. Gels were arrayed with samples from individuals attending a nest adjacent to one another. If all hybridization bands observed for nestlings could have been inherited from the primary pair, we concluded that the primary pair was the parents. If, however, a hybridization band could be accounted for only by parentage by a nest attendant, we concluded that an extra-pair mating had occurred. There was only one adult female at each nest. The male that was most dominant and exhibited the greatest paternal investment (Tingay 2000) was considered the male of the primary pair.

DNA band-sharing (Bruford et al. 1992) was calculated as $S = 2n_{xy}/(n_x + n_y)$, where n_{xy} = the number of bands shared by both individuals, n_x = the total number of bands exhibited by individual x, and n_y = the total number of bands exhibited by individual y. Band-sharing was estimated for all combinations of individuals in this study. The range of S for known parent-offspring combinations provided a quantitative expectation of how many bands must be shared before a hypothesis of familial relatedness was supported.

RESULTS

Parentage Assessment of Nestlings and Juveniles. DNA fingerprinting techniques were used to

assess relatedness of 17 eagles at four nests. Two enzymes (*Hae*III and *Rsa*I) produced clearly interpretable results yielding a total of 34 bands scored, 24 of which were variable and 10 invariant (Table 1). Of the 24 variable bands, six were informative in determining one or more possible parents for the two nestlings at Soamalipo 2; three for the juvenile at Befotaka 3; and seven for the nestling at Befotaka 2. Blood samples were available only for adults at Ankerika 4. A nest-by-nest assessment of parentage is presented below.

Befotaka 2. Female 121, α male 118, and β male 8 attended the nest. Nestling 47 shared three variant *Hae*III and one variant *Rsa*I hybridization bands with adult female 121, and two variant *Hae*III and one variant *Rsa*I hybridization bands with β male 8, suggesting that subordinate β male 8 was the father of the nestling 47, and not α male 118.

Befotaka 3. Female 6, potential α male 48, and potential α male 150 attended this nest. Juvenile 128 shared one *Hae*III band and one *Rsa*I band with adult female 6. Banding records show that juvenile 128 fledged from this nest in 1998. Although band sharing showed it unlikely that either adult male at the nest in 1999 (48 and 150) was the father, it is highly probable that the adult female at the nest is the mother ($S = 0.95$ is the highest value in the study, female 6 has been recorded at this nest site every year since 1993, and no other female has been recorded at this nest).

Soamalipo 2. Female 103, α male 5, β male 136, and γ male 30 attended this nest. Nestling 68 shared one *Hae*III band with adult female 103 and two *Rsa*I bands with γ male 30. Nestling 00 shared one *Hae*III and one *Rsa*I band with adult female 103 and one *Hae*III and three *Rsa*I bands with γ male 30. The apparent father of both nestlings is subordinate γ male 30.

Relatedness Estimates of All Adults Within and Between Nests. Among 136 pairwise comparisons, band-sharing among individuals ranged from 0.58–0.95, with a mean value of 0.79. Partitioning pairwise band-sharing into within- and between-nest components showed no difference (mean $S = 0.80$ within nests and 0.79 between nests). After accounting for eight known first-order relative pairs (parent-offspring, full-sibling), band-sharing was higher among first-order relatives ($\bar{x} = 0.87$, range = 0.82–0.95) than overall ($\bar{x} = 0.79$; Table 2). Using these findings, relatedness among adults attending nests (male-male, male-female) was determined (Table 2).

Ankerika 4. Band-sharing values suggested a potential first-order relationship between female 113 and α male 31, but not between the female 113 and β male 34. Band-sharing suggested that the males were unrelated.

Befotaka 2. Band-sharing values did not support a first-order relationship between the female and either male, nor between males. β male 8 had two bands not shared with any individual within the study population; trapping records indicate that β male 8 fledged from the Befotaka 3 nest in 1993.

Befotaka 3. Band-sharing values indicated a potential first-order relationship between female 6 and male 150, but not between female 6 and male 48. Band-sharing suggested that the males were unrelated.

Soamalipo 2. Band-sharing values indicated a potential first-order relationship between female 103 and α male 5, but not between female 103 and the two subordinate males (β 136 and γ 30). Band-sharing between α male 5 and γ male 30 indicated a potential first-order relationship.

Relatedness estimates between nests. Comparing among nests, we observed high band-sharing values between female 121 (Befotaka 2) and female 103 (Soamalipo 2), male 5 (Soamalipo 2) and male 48 (Befotaka 3), and between male 34 (Ankerika 4) and female 6 (Befotaka 3), suggesting potential first-order relatedness between these pairs of adults.

DISCUSSION

Subordinate males may have fathered all nestlings in this study. At Soamalipo 2, one subordinate male appeared to have fathered both nestlings; however, because α male 5 and γ male 30 are close relatives, and because of missing data for α male 5, we cannot exclude α male 5 as a possible father of one or both nestlings. At all nests, paternity by subordinates could have occurred by chance, as all attending males copulated with the female (Tingay 2000). Paternity by subordinates was surprising given that dominant males invested more energy to the nesting attempt than subordinate males (Tingay 2000). This level of dominant male investment may be explained by the apparent first-order relatedness of the female and the dominant male at three of four nests (Ankerika 4, Befotaka 3, and Soamalipo 2). Because 50% of alleles are shared with a first-order relative, and 25% with an offspring of a first-order relative, then shared alleles are transmitted to the next generation if a first-

Table 1. DNA fingerprinting hybridization bands (Jeffreys 33.15 probe) observed for individual Madagascar Fish-Eagles. Bands are designated by enzyme used (H = *Hae*III or R = *Rsa*I) and molecular weight of bands in kilobase pairs. Sex and rank for individuals is indicated (F = female, αM = alpha male, βM = beta male, γM = gamma male, NSL = nestling, JUV = juvenile).

	ANKERIKA 4[a]			BEFOTAKA 2[b]				BEFOTAKA 3[c]				SOAMALIPO 2[d]					
INDIVIDUAL	F 113	αM 31	βM 34	F 121	αM 118	βM 8	NSL 47	F 6	αM? 150	αM? 48	JUV 128	F 103	αM 5	βM 136	γM 30	NSL 68	NSL 00
Bands																	
H 16.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H 10.7				+			+			+				+	+		
H 8.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H 7.3		+		+	+	+	+	+	+		+	+	+	+	+	+	
H 6.5		+				+	+	+			+						
H 6.0			+			+	+			+			+				
H 5.7	+	+			+				+					+	+		
H 5.6			+						+	+							
H 5.2					+									+			
H 4.9		+							+			+		+			
H 4.7						+											
H 4.5	+	+	+	+			+	+	+	+	+	+	+		+	+	+
H 3.9	+	+	+	+	+	+	+	+	+	+	+	+	+			+	+
H 3.6			+	+	+		+	+			+	+					+
H 3.2	+	+	+	+		+	+	+	+			+				+	+
H 2.9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H 2.7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H 2.6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H 2.2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H 1.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H 1.4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H 1.0	+	+		+			+			+		+	+	+	+	+	+
H 0.9	+		+		+	+	+	+	+	+	+		+		+		+
R 12.0	+	+	+	+	+	+	+	+	+	+	+	+	?	+	+	+	+
R 5.2	+	+				+	+						?				
R 5.0			+	+	+		+	+	+			+	?	+		+	+
R 4.7			+					+			+		?		+	+	+
R 4.5						+							?				
R 4.4	+		+		+	+	+					+	?				+
R 4.2								+					?		+		+
R 3.3	+	+	+	+	+	+	+	+	+	+	+	+	?	+	+	+	+
R 1.5	+	+		+						+		+	?	+	+	+	+
R 1.4	+		+		+	+	+	+	+	+	+		?		+	+	+
R 1.2	+	+	+	+	+	+	+	+	+	+	+	+	?	+	+	+	+
Total No. bands per individual	21	21	22	20	20	22	26	21	21	20	19	21	14	19	21	20	23

[a] Fifteen bands are variable at Ankerika 4 (H 7.3, H 6.5, H 6.0, H 5.7, H 5.6, H 4.9, H 3.6, H 1.0, H 0.9, R 5.2, R 5.0, R 4.7, R 4.4, R 1.5, R 1.4). All other bands are invariant.

[b] Of the variable bands at Befotaka 2, six are shared between the nestling and the female (H 10.7, H 4.5, H 3.6, H 1.0, R 5.0, R 1.5); six are shared between the nestling and the beta male (H 6.5, H 6.0, H 0.9, R 5.2, R 4.4, R 1.4); one is shared between the nestling, female, and beta male (H 3.2); and four are variable but are not observed in the nestling (H 5.7, H 5.2, H 4.7, R 4.5). All other bands are invariant.

[c] Of the variable bands at Befotaka 3, four are shared between the juvenile and the female (H 7.3, H 6.5, H 3.6, R 4.7); and ten are variable but are not observed in the juvenile (H 10.7, H 6.0, H 5.7, H 5.6, H 4.9, H 3.2, H 1.0, R 5.0, R 4.2, R 1.5). All other bands are invariant.

[d] Of the variable bands at Soamalipo 2, four are shared between nestling 68 and the female (H 7.3, H 3.9, H 3.2, R 5.0); two are shared between nestling 68 and the gamma male (R 4.7, R 1.4); and one is shared between nestling 68, the female, and the gamma male (H 4.5). Five bands are shared between nestling 00 and the female (H 3.9, H 3.6, H 3.2, R 5.0, R 4.4); four are shared between nestling 00 and the gamma male (H 0.9, R 4.7, R 4.2, R 1.4); and one is shared between nestling 00, the female, and the gamma male (H 4.5). Five are variable but are not observed in either nestling (H 10.7, H 6.0, H 5.7, H 5.2, H 4.9); and all other bands are invariant.

Table 2. Pairwise band-sharing values for multilocus DNA fingerprints (*Hae*III and *Rsa*I), using Jeffreys' 33.15 probe (Jeffreys et al. 1985), among Madagascar Fish-Eagles. Sex and rank for individuals is indicated (F = female, αM = alpha male, βM = beta male, γM = gamma male, NSL = nestling, JUV = juvenile). Bold is intended to allow easier reading of within- and between-nest comparisons, all within-nest comparisons are contained in bold triangles, all comparisons between-nests are contained in bold or non-bold squares.

			ANKERIKA 4			BEFOTAKA 2				BEFOTAKA 3				SOAMALIPO 2					
			F	αM	βM	F	αM	βM	NSL	F	αM?	αM?	JUV	F	αM	βM	γM	NSL	NSL
	INDIVIDUAL		113	31	34	121	118	8	47	6	150	48	128	103	5	136	30	68	0
Ankerika 4	F	113																	
	αM	31	**0.86**																
	βM	34	**0.79**	**0.65**															
Befotaka 2	F	121	0.78	0.83	0.76														
	αM	118	0.78	0.68	0.81	**0.75**													
	βM	8	0.79	0.74	0.77	**0.67**	**0.76**												
	NSL	47	0.85	0.81	0.83	**0.87**	**0.78**	**0.83**											
Befotaka 3	F	6	**0.76**	**0.76**	**0.88**	0.83	0.83	0.79	**0.85**										
	αM?	150	**0.81**	**0.81**	**0.84**	0.78	0.83	0.74	**0.77**	**0.86**									
	αM?	48	**0.83**	**0.73**	**0.81**	0.80	0.70	0.71	**0.83**	**0.73**	**0.78**								
	JUV	128	**0.75**	**0.75**	**0.83**	0.77	0.82	0.78	**0.80**	**0.95**	**0.80**	**0.77**							
Soamalipo 2	F	103	0.81	0.86	0.79	**0.93**	**0.78**	**0.70**	0.85	0.81	0.81	0.73	0.75						
	αM	5	0.86	0.80	0.83	**0.83**	**0.79**	**0.83**	0.88	0.86	0.80	0.90	0.86	**0.83**					
	βM	136	0.70	0.80	0.59	**0.82**	**0.77**	**0.59**	0.71	0.65	0.75	0.72	0.63	**0.80**	**0.71**				
	γM	30	0.81	0.76	0.70	**0.78**	**0.73**	**0.65**	0.77	0.81	0.76	0.83	0.80	**0.71**	**0.86**	**0.80**			
	NSL	68	0.83	0.83	0.81	**0.90**	**0.75**	**0.71**	0.83	0.88	0.83	0.80	0.82	**0.88**	**0.89**	**0.77**	**0.83**		
	NSL	0	0.86	0.73	0.89	**0.84**	**0.79**	**0.71**	0.86	0.91	0.77	0.79	0.81	**0.86**	**0.86**	**0.67**	**0.82**	**0.88**	

order relative reproduces successfully. At Soamalipo 2, the dominant male gained an additional genetic advantage by having two potential first-order relatives at the nest (the female and the γ male). It would be advantageous to be a male at the same nest as a brother, because if either mated successfully, then shared genes are transmitted to the next generation. Although a strategy of assisting reproductive efforts of close relatives may be advantageous for some Madagascar Fish-Eagles, apparently it is not the only strategy in use. At Befotaka 2, the dominant male was not the father, and nor was he a first-order relative of either the female or the subordinate male.

At Befotaka 3, a juvenile female did not disperse. This is the first observed instance of a female nestling from a previous year remaining at a nest (Rafanomezantsoa 1997). Here, delayed dispersal was not associated with observed helping activity, yet the female juvenile was tolerated at the nest. Although inconclusive, our findings do not exclude the delayed dispersal hypothesis.

Between-nest relatedness comparisons revealed that some adults had a potential close relative (parent-offspring or full-sibling) at another nest within the study area. This suggests that first-order relatives (excluding nestlings) are as likely to be found among nests as within a nest.

We are currently investigating the full range of breeding strategies in the Madagascar Fish-Eagle. We intend to determine whether this species exhibits genetic monogamy or polyandry by extending our sample size and duration of study. Studies of another cooperative polyandrous raptor species, the Galápagos Hawk (*Buteo galapagoensis*) has revealed mixed paternity at nests over two consecutive breeding seasons (Faaborg et al. 1995). However, the dominance hierarchy we have observed among cooperative fish-eagles has not been documented among Galápagos Hawks, which may or may not influence the occurrence of genetic monogamy within polyandrous groups of Madagascar Fish-Eagles. If delayed dispersal is obligatory in this species, recolonization of unoccupied habitats may have to be promoted by active conservation measures, such as the translocation of individuals from other areas. Additionally, copulation by closely-related pairs, as observed in this study, suggests that the effects of inbreeding may have to be considered in conservation planning. For example, if first-order relatives are found to be producing offspring, conservation managers may wish to target some of those specific individuals as likely candidates for translocation, in order to reduce the probability of further inbreeding and to create an opportunity for outbreeding with other, genetically dissimilar, individuals.

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