J. Raptor Res. 41(1):45–49

USING MOLECULAR SEXING TO ASSESS FIELD-BASED SEXING TECHNIQUES IN THE MADAGASCAR FISH-EAGLE

RUTH E. TINGAY[1]
School of Geography, University of Nottingham, University Park, Nottingham, NG7 2RD, U.K.

MELANIE CULVER
Arizona Cooperative Fish & Wildlife Research Unit (USGS), 104, Biological Sciences East, University of Arizona, Tucson, AZ 85721 U.S.A.

RICHARD T. WATSON
The Peregrine Fund, 5668 West Flying Hawk Lane, Boise, Idaho, ID 83709 U.S.A.

KEY WORDS: *Madagascar Fish-Eagle*; Haliaeetus vociferoides; *copulatory position*; *mass*; *molecular sexing*; *polyandrous*; *sexual dimorphism.*

The ability to accurately sex individuals is a fundamental tool in many avian ecology and conservation studies. Skewed sex ratios may influence effective population size and ultimately genetic erosion (Lande and Barrowclough 1996); thus, sex information has provided important guidance criteria for the management of endangered bird species including the Kakapo (*Strigops habroptilus*; Robertson et al. 2000) and the Poo-uli (*Melamprosops phaeosoma*; Groombridge et al. 2004). Sex determination of adults may be easily assigned in some raptor species by observing obvious sexually dimorphic traits such as plumage (e.g., Northern Harrier, *Circus cyaneus*, Watson 1977) or size (e.g., Eurasian Sparrowhawk, *Accipiter nisus*, Newton 1986). However, in other species, adult sexual dimorphism is absent (e.g., Himalayan Griffon, *Gyps himalayensis*, Ferguson-Lees and Christie 2001) or, not very pronounced (e.g., Slender-billed Kite, *Rostrhamus hamatus*, Ferguson-Lees and Christie 2001), so a different technique must be used to determine sex. Several molecular sexing techniques have been developed (Griffiths et al. 1998, Fridolfsson and Ellegren 1999) that have demonstrated that the female chromosome (CHD1W) is consistently smaller than the male chromosome (CHD1Z) in birds and thus sex can be reliably identified by the relative size of an individual's DNA subjected to polymerase chain reaction (PCR).

The Madagascar Fish-Eagle (*Haliaeetus vociferoides*) is a critically endangered island endemic (IUCN 2006) with a known population last documented in 1995 of 222 individuals (Rabarisoa et al. 1997). Accurate sex assignment in this species was required to identify its multiple social and breeding strategies (including monogamy, polyandry, polygyny, polygynandry, and homosexuality), to model population dynamics, and to assist in conservation planning (Tingay 2005). As male and female Madagascar Fish-Eagles have monomorphic plumage (Langrand 1990) and slight reversed sexual size dimorphism that is not easily discerned in the field (Tingay 2000), other field-based techniques have been employed to tentatively assign sex. These have included mass measurements (females are thought to be heavier than males; Tingay 2000), observed copulatory position (Tingay 2000) and the dimorphic pitch of calls (Rafanomezantsoa 2000). The Madagascar Fish Eagle is vocally conspicuous, frequently yelping "ko ko koy koy koy," either as a solo call or in duet (Langrand and Meyburg 1989). Although the pitch dimorphism is subtle, males are thought to have a higher pitch than females (Rafanomezantsoa 2000). Here we report the use of molecular sexing to verify the accuracy of these field-based techniques to assign sex in adult and subadult Madagascar Fish-Eagles.

STUDY AREA AND METHODS

Study Area. The study was conducted in the Manambolomaty River floodplain (19°00'S, 44°30'E) in the Antsalova region of western Madagascar, ca. 300 km west of the capital, Antananarivo. The habitat is dominated by tropical, deciduous, dry forest containing several freshwater lakes (with areas of 3.1–4.9 km^2) that supported 11 Madagascar Fish-Eagle territories (Rabarisoa et al. 1997).

Field Methods. From May to September 1999–2001, we trapped, weighed, bled, and color-banded 43 Madagascar Fish-Eagles (38 adults, 5 subadults) using either a noosed fish (Wiersma et al. 2001) or a noose carpet (Bloom 1987). Eagles were weighed with a 4-kg Pesola® spring balance and banded with a uniquely numbered embossed aluminum leg band and a series of colored plastic or colored aluminum leg bands for individual identification. We assumed that individuals weighing <2750 g were males and those weighing >2750 g were females, based on data from a previous study (Tingay 2000). Blood (0.25–0.75 ml) was taken from the brachial vein, immediately placed in 4.5 ml

[1] Present address: Natural Research Ltd., Burn O'Bennie Road, Banchory, AB31 5ZU, Scotland; Email address: dimlylit100@hotmail.com

of lysis buffer (100 mM, pH 8.0, Tris HCl, 100 mM EDTA, 10 mM NaCl, 0.5% SDS) in a polypropylene tube, labelled and stored at ambient temperature. Subsequent behavioral observations of color-banded individuals (>2000 hr of observation) included the tentative assignment of sex based on the individual's call pitch and, for adults, their observed copulatory position (assumed males in the upper position). As we were unable to confirm the sex of any of the Madagascar Fish-Eagles, we used blood samples from the closely-related African Fish-Eagle (*Haliaeetus vocifer*; Lerner and Mindell 2005) to serve as positive controls. Blood was taken from the brachial vein of eight African Fish-Eagles in a captive-breeding program at the National Birds of Prey Centre, U.K. (and thus of known sex) and stored as above.

Molecular Methods. Genomic DNA was extracted from whole blood samples using a QIAamp® DNA Blood Mini Kit (Qiagen Inc., Valencia, CA U.S.A.). Water served as negative contamination control sample for PCR amplifications. All PCR reactions were performed in a 10 µl volume in a thermocycler (Eppendorf Mastercycler Gradient®) using Eppendorf Mastermix Taq® at 1 X concentration, 0.1 µM of each primer (2550F and 2718R; Fridolfsson and Ellegren 1999), 1.75 mM $MgCl_2$, 1% BSA, and 2 µl of 10 ng template DNA. The thermal profile was one cycle of 94°C for 2 min, followed by a touch-down scheme where annealing temperature was lowered 1°C per cycle from 60°C to 50°C, followed by 35 cycles at 50°C. Denaturation was at 94°C for 30 sec, annealing for 30 sec and extension at 72°C for 40 sec. A final extension step was performed at 72°C for 5 min, then the reaction was held at 4°C indefinitely. PCR amplification products were stained with ethidium bromide and subjected to electrophoresis on a 1% agarose gel, in TBE buffer (Tris, Borate, EDTA), at 60 V for 120 min before visualization under ultra violet light.

RESULTS

Field-based Sex Assignment. We assumed, based on body mass, that 27 of the trapped eagles were males and 16 were females (Table 1, 2). We observed the copulatory position of 35 marked adults and identified these as 22 males and 13 females (Table 2). Note was made of the auditory call pitch of 40 marked adults and subadults (i.e., either high or low pitch) and sex was assigned as 25 male and 15 female (Table 2).

Molecular-based Sex Assignment. The eight African Fish-Eagle control samples all produced amplifiable products. The visualized CHD1Z fragments of the three known males were scored at 766 base pairs (bp) and the visualized CHD1W fragments of the five known females were scored at 500 bp. The 43 Madagascar Fish-Eagle samples all produced amplifiable products which were equally as distinguishable by length polymorphism, comprising 27 males (766 bp) and 16 females (500 bp).

For the 43 Madagascar Fish-Eagles for which sex was assigned by body mass, the sex identified using molecular sexing was identical. For the 40 Madagascar Fish-Eagles for which sex was assigned by the pitch of the call, the sex identified using molecular sexing was identical. For the 35 eagles for which sex was assigned based on their copulatory position, the molecular sexing results matched 34 individuals. Madagascar Fish-Eagle #0120 was incorrectly sexed in the field as a female (Table 2), based on the bird's lower position during an attempted copulation with Madagascar Fish-Eagle #0007. These two eagles were presumed to be a breeding pair as they held a territory and were regularly observed in nest-building activities (Tingay 2005). However, the sex of Madagascar Fish-Eagle #0120 was uncertain as he weighed only 2500 g and had a high-pitched call. Molecular sexing revealed that both eagles were male.

Table 1. Mean mass (g with standard deviation) of assumed male and female adult and subadult Madagascar Fish-Eagles.

ASSUMED SEX	*N*	MEAN	RANGE
Male	27	2377 ± 134	2200–2700
Female	16	3113 ± 169	2800–3500

DISCUSSION

The molecular sexing technique of Fridolfsson and Ellegren (1999) was suitable for use in Madagascar Fish-Eagles. We chose these primers in preference to those used by Griffiths et al. (1998) because, although those have previously been successfully used to determine sex in several raptor species (Norris-Caneda et al. 1998), the primers developed by Griffiths et al. (1998) can produce less distinguishable length polymorphism in the amplified products which may lead to scoring error (see Dawson et al. 2001). In our study, the size of male and female CHD1 fragments in both *Haliaeetus* species (Madagascar and African Fish-Eagle) differed from those predicted by Fridolfsson and Ellegren (1999), but they followed the predicted pattern of consistent size differentiation. The *Haliaeetus* male CHD1Z fragments were 766 bp (compared to the 600–650 bp predicted) and the *Haliaeetus* female CHD1W fragments were 500 bp (compared to the 400–450 bp predicted). As the African Fish-Eagle individuals were of known sex and followed the same pattern as the Madagascar Fish-Eagle individuals, the results for the Madagascar Fish-Eagles were considered reliable. Further support for this interpretation is provided by a recent study of the closely related White-tailed Sea Eagle (*Haliaeetus albicilla*; F. Hailer pers. comm.), in which the amplified PCR products in both sexes were also larger than those predicted by Fridolfsson and Ellegren (1999).

Our results demonstrated the reliability of two of the three field-based techniques for assigning sex in adult and subadult Madagascar Fish-Eagles. Molecular sexing confirmed that males and females could be accurately distinguished by the pitch of their call (males are higher), less

Table 2. Sex assignment of 43 Madagascar Fish-Eagle adults and subadults by body mass, copulatory position, pitch of call and DNA analysis. Subadults are identified by a * before the ID number. The individual whose sex was incorrectly assigned based on copulatory position is italicized.

Fish-Eagle ID (Band #)	Body Mass (g)	Sex by Mass	Sex by Copulatory Position	Pitch of Call	Sex by Pitch of Call	Sex by DNA
0138	3200	F	F	LOW	F	F
0142	2300	M	M	HIGH	M	M
0062	2300	M	M	HIGH	M	M
*0139	2800	F	—	LOW	F	F
0013	3000	F	—	—	—	F
0012	2350	M	—	—	—	M
0110	3100	F	F	LOW	F	F
0137	2400	M	M	HIGH	M	M
0028	2400	M	M	HIGH	M	M
0020	3100	F	F	LOW	F	F
0033	2400	M	M	HIGH	M	M
0011	2200	M	M	HIGH	M	M
0141	2200	M	M	HIGH	M	M
*0102	2350	M	—	—	—	M
0113	3000	F	F	LOW	F	F
0031	2500	M	M	HIGH	M	M
0034	2300	M	M	HIGH	M	M
0121	3015	F	F	LOW	F	F
0118	2550	M	M	HIGH	M	M
*0146	2200	M	—	HIGH	M	M
0006	2900	F	F	LOW	F	F
0048	2400	M	M	HIGH	M	M
0150	2350	M	M	HIGH	M	M
*0042	3000	F	—	LOW	F	F
0107	2200	M	—	HIGH	M	M
*0128	3200	F	—	LOW	F	F
0111	3200	F	F	LOW	F	F
0115	3100	F	F	LOW	F	F
0131	2450	M	M	HIGH	M	M
0129	2300	M	M	HIGH	M	M
0143	3000	F	F	LOW	F	F
0149	2600	M	M	HIGH	M	M
0021	2300	M	M	HIGH	M	M
0117	3500	F	F	LOW	F	F
0026	2600	M	M	HIGH	M	M
0120	***2500***	***M***	***F***	***HIGH***	***M***	***M***
0007	2200	M	M	HIGH	M	M
0103	3300	F	F	LOW	F	F
0136	2350	M	M	HIGH	M	M
0005	2700	M	M	HIGH	M	M
0030	2500	M	M	HIGH	M	M
0116	3300	F	F	LOW	F	F
0104	2300	M	M	HIGH	M	M

confidently by their weight (females are heavier), and not confidently by their copulatory position. Determination of sex by vocalizations is a technique employed in studies of other sexually monomorphic avian species, such as Northern Spotted Owls (*Strix occidentalis caurina*; Blakesley et al. 1990), Whooping Cranes (*Grus americana*; Carlson and Trost 1992), Manx Shearwaters (*Puffinus puffinus*; Brooke 1978) and Western Screech-Owls (*Megascops kennicottii*; Herting and Belthoff 2001). Determining sex by vocalization is a particularly useful tool for studies of

highly endangered species such as the Madagascar Fish-Eagle because it can minimize the handling stress associated with traditional sexing techniques such as the collection of morphometric data. The use of weight dimorphism to determine sex in Madagascar Fish-Eagles obviously does not minimize the associated handling stress, but nevertheless, is a useful secondary tool in the absence of vocalization data. Caution should be applied, however, if using only this technique to determine sex because there is little difference (100 g) between the heaviest male and the lightest female. Because this difference could easily lead to an incorrect sex assignment if the mass of the eagle is inaccurately recorded, we recommend using supportive molecular sexing for eagles within the overlap range.

The use of observed copulatory position to determine sex in Madagascar Fish-Eagles was unreliable in one out of 35 instances, where a male-male mounting was observed. Male-male mountings or reverse male mountings (i.e., the female mounts the male) are not infrequent in other sexually monomorphic polyandrous raptors, such as the Bearded Vulture (*Gypaetus barbatus*; Bertran and Margalida 2003, 2006). Male reversed sexual behaviour has also been reported in a polyandrous trio of Spanish Imperial Eagles (*Aquila adalberti*; González et al. 2006). Given the unique array of cooperatively breeding social groups and the small population size of Madagascar Fish-Eagles (Tingay 2005) and the increasing documentation of unusual copulatory behaviour amongst other polyandrous raptors, we recommend that copulatory position not be used as a primary indication of sex in sexually monomorphic polyandrous raptor species.

USO DE MÉTODOS MOLECULARES PARA EVALUAR LAS TÉCNICAS DE CAMPO PARA DETERMINAR EL SEXO EN *HALIAEETUS VOCIFEROIDES*

RESUMEN.—Las técnicas de campo para determinar el sexo de individuos adultos de la especie *Haliaeetus vociferoides* han incluido datos del peso corporal, la frecuencia de las vocalizaciones y la posición durante la cópula. Pusimos a prueba la confiabilidad de dichas técnicas empleando métodos moleculares de determinación del sexo en 43 individuos marcados. Demostramos la confiabilidad del uso del peso corporal y las frecuencias de las vocalizaciones para determinar el sexo con exactitud en el campo, y la poca confiabilidad del uso de la postura durante la cópula.

[Traducción del equipo editorial]

ACKNOWLEDGMENTS

We thank the Madagascar Direction des Eaux et Fôrets for collaboration and for issuing the research permits allowing us to trap eagles and obtain blood samples. Special thanks to Loukman Kalavaha, Adrien Batou, Martin Gilbert, Brian Bourke and Richard Seaton for assistance in the field. We are grateful to Victor David for providing valuable technical advice during the lab work. Blood samples from captive African Fish-Eagles were kindly provided by Jemima Parry-Jones (National Birds of Prey Centre, U.K.), Campbell Murn (Hawk Conservancy, U.K.), Maria Crane (Zoo Atlanta, U.S.A.) and Jim Chick (U.K.). Thanks to Lloyd Kiff and Travis Rosenberry who provided material for the literature review. This study was funded by grants to RET from The Peregrine Fund, University of Nottingham, Hawk Mountain Sanctuary Project Soar, International Osprey Foundation, Morley Nelson Fellowship, and Raptor Research Foundation Leslie Brown Memorial Fund. We thank Phil Whitfield, Mike McGrady, Frank Hailer, Carol McIntyre and three anonymous reviewers for their thoughtful comments on an earlier draft of the manuscript.

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Received 19 June 2006; accepted 9 January 2007
Associate Editor: Carol L. McIntyre