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SEXING BONELLI'S EAGLE NESTLINGS: MORPHOMETRICS VERSUS MOLECULAR TECHNIQUES

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ABSTRACT.—We report details of two PCR-based molecular sexing techniques for the Bonelli's Eagle (*Hieraaetus fasciatus*) and evaluate the reliability of morphometric measurements to predict the sex of nestlings in the field. Blood samples taken from 63 nestlings in southwest Portugal (1994–99) were analyzed using the intron polymorphism method (M1), and 56 of these were also analyzed with the single-strand conformation polymorphism approach (M2). Contamination or poor preservation of samples precluded one sex determination with M1 and six others with M2. Sexing by both methods was concordant for 98.0% of samples. Linear discriminant analysis was used to determine whether any single variable or combination could provide reliable sex determinations, using 10 body measurements from 43 nestlings aged 35–50 d, sexed unambiguously by both molecular methods. Models were evaluated by cross-validation of the original data and from the classification of an external sample ($N = 12$). Females were significantly larger than males. The greatest separation between sexes occurred in body mass, but differences were also noted in tarsus diameter and the lengths of the hind claw, foot, culmen, and forewing; no differences were detected in the lengths of tarsus, fore claw, seventh primary, and central tail feather. A discriminant model including body mass, hind claw length, and age provided the maximum separation between sexes and it correctly sexed 96% of the nestlings. A model including tarsus diameter, hind claw, and age showed similar accuracy. Both models were satisfactory in determining the sex of nestling Bonelli's Eagles between the ages of 35–50 d in the field, but combination with molecular techniques may be preferable in studies requiring absolute precision for every individual handled.

KEY WORDS: *Bonelli's Eagle*; Hieraaetus fasciatus; *CHD1 gene*; *sex determination*; *Portugal.*

Determinación del sexo en pollos de *Hieraetus fasciatus:* medidas morfométricas versus técnicas moleculares

RESÚMEN.—Exponemos detalles de dos métodos moleculares para la determinación de sexo de águila-azor perdicera (*Hieraaetus fasciatus*) y evaluamos la validez de algunas medidas morfométricas para sexar pollos en el campo. Se analizaron muestras de sangre de 63 pollos del suroeste de Portugal (1994–99) mediante el "intron polymorphism method" (M1) y 56 de ellas también por el "single-strand conformation polymorphism method" (M2). La determinación del sexo no fue posible por el método M1 en

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una muestra y por el M2 en otras seis, a causa de la contaminación y deterioro de las muestras. Los sexados obtenidos por los dos métodos moleculares fueron concordantes en el 98.0% de las muestras. Evaluamos qué variables o combinaciones de variables permiten la máxima separación entre los sexos, utilizando un análisis discriminante linear en diez medidas externas, tomadas en los 43 individuos con edades entre 35–50 días, en que ambos métodos moleculares han producido resultados coincidentes. Los modelos fueron evaluados por validación cruzada de los datos originales y por la clasificación de una muestra adicional de validación ($N = 12$). Las hembras fueron significantemente mayores que los machos en peso, diámetro del tarso, garra posterior, pata, pico y antebrazo; no se encontraron diferencias en la longitud del tarso, garra anterior, séptima primaria y pluma central de la cola. El modelo que incluye el peso, garra posterior y edad, clasificó correctamente el sexo de 96% de individuos. Un modelo que incluye el diámetro del tarso, garra posterior y edad, obtuvo semejante grado de exactitud. Los dos modelos permiten la correcta clasificación del género de los pollos de águila perdicera con 35–50 días de edad, pero la combinación con métodos moleculares es preferible para una determinación absolutamente precisa del sexo de todas las aves.

[Traducción de autores]

In recent years, a number of methods have been proposed for sexing birds using DNA markers (e.g., Ellegren and Shelton 1997, Griffiths et al. 1998, Cortés et al. 1999). This has prompted a renewed scientific interest in a number of topics requiring the rigorous determination of sex and thus facilitating the investigation of species showing only slight, if any, sexual dimorphism. These techniques have been particularly helpful in addressing questions related to avian offspring sex ratios (Ellegren and Shelton 1997), owing to difficulties in sexing nestlings by conventional methods (Clutton-Brock 1986).

Raptors tend to be sexually dimorphic in size and external morphometry is commonly used in sex identification (e.g., Ferrer and de le Court 1992). In many instances, however, there is considerable overlap between males and females in external characteristics and significant rates of misclassification may occur (Morrison and Maltbie 1999). Additional errors are likely if physical condition affects body measurements, eventually causing diseased or undernourished birds to diverge from the pattern typical for their sex. Therefore, it is important that studies using external morphological characters for sex determination are able to document, in detail, the error rates associated with the use of these methods. Reliable sex determination using molecular techniques provides the means to assess the validity of the methods based on external morphology and to identify the morphological characteristics most adequate for sex determination (King and Griffiths 1994, Morrison and Maltbie 1999).

The Bonelli's Eagle (*Hieraaetus fasciatus*) is a medium-sized raptor, whose numbers and range have declined markedly in Europe, where it is restricted to the Mediterranean region (Rocamora 1994). Adult females are significantly larger than males (Parellada, 1984), and this may also be the case for nestlings (Mañosa et al. 1995). However, it is not known whether size alone is reliable to determine sex in Bonelli's Eagle nestlings. The objectives of this study were to adapt distinct molecular techniques to identify the sex of Bonelli's Eagles following Griffiths et al. (1998) and Cortés et al. (1999) and to derive and evaluate methods for using external morphometric measurements to determine accurately the sex of nestlings in the field.

MATERIALS AND METHODS

Field Sampling. Data were collected as part of a long-term study on the Bonelli's Eagle in the uplands of Algarve and western Alentejo in southern Portugal. This is a hilly landscape covered primarily by cork oak (*Quercus suber*) woods, dense Mediterranean scrub and eucalyptus (*Eucalyptus globulus*) plantations, with sparse human occupation. Bonelli's Eagles breed primarily in large cork oaks, eucalyptus, and pine trees (*Pinus pinaster*) (Palma 1994). We have monitored this population regularly since 1993, checking breeding performance and ringing the nestlings each year. During 1994–99, external morphometric measurements and blood samples were taken from 63 nestlings to determine sex. To minimize the risk of premature fledging (Grier and Fyfe 1987), sampling was limited to nestlings <50 d of age.

Molecular Methods. Blood samples were collected from the brachial vein of Bonelli's Eagle nestlings. One drop of blood (± 50 µl) from each nestling was conserved in "Queen's" lysis buffer at 4°C, and later used for the extraction of genomic DNA as described by Seutin et al. (1991). A second portion (1–2 ml) of blood was conserved in a Lithium-heparinized sterile tube and maintained at 4°C, and subsequently separated in serum and red cell fractions by centrifugation. The red cell fraction was conserved in a glycerol solution at −20°C, and was used for both the analysis of allozyme variability (Cardia et al. 2000) and DNA extraction with a QIAamp Tis-

sue Kit (QIAGEN, Gmbh, Hilden, Germany). The two DNA samples, each one analyzed in a different laboratory with a distinct molecular method, were then used in a double blind scheme for sex determination. Both molecular methods are based on the PCR amplification of a portion of the CHD1 gene (Ellegren and Sheldon 1997, Griffiths et al. 1998), which is known to be located in a region of the sex chromosomes that is not affected by recombination (Fridolfsson et al. 1998). Molecular sexing techniques were tested on blood samples taken from four captive adult Bonelli's Eagles (2 M, 2 F). The first DNA sample was analyzed with the intron polymorphism method (M1), which is based on the amplification of the second intron of the CHD1 gene, the size of which is different between males and females in most bird species (Griffiths et al. 1998). The primers used for this method were chosen so that they would amplify the same region as primers P2 and P8 described by Griffiths et al. (1998). They were designed from an alignment of 16 CHD1 sequences of birds (CHD1-Z and -W denoting the copy of the Z and W chromosomes; accession numbers: FAY12939, FAY12942, ECY12940-1, AF006661-2, AF006659-60, AF060701, AF128256, AF128255, AF128254 and AF077936-7) and mammals (L10410, AF006513) found in the Genebank database (http://www.ncbi.nlm.nih.gov/) in order to ensure the success of amplification in the Bonelli's Eagle. The primers were located in the two exons bordering the target intron and corresponded to the regions of highest homology between the aligned sequences (from 5′ to 3′, numbers correspond to the numbering of the *M. musculus* sequence): CCAAGRATGAGAAACTGTGC (3375–3395) and TCTGCATCRCTAAATCCTTT (3760–3740). Radioactive PCR reactions contained about 50 ng DNA, 1X reaction buffer supplied by the manufacturer, 1.5 mM $MgCl_2$, 100 μM dATP, 60 μM each of the other dNTPs, 10 μM each primer, 0.15 unit of *Taq* polymerase (Gibco BRL, Life Technologies Inc., Gaithersburg, MD U.S.A.) and 0.16 μl [α-^{35}S]dATP (12.5 mCi/ml, 1250 Ci/mmol). The amplification was performed in a Stratagene Robocycler (Stratagene Cloning Systems, La Jolla, CA U.S.A.) and cycles consisted of 1.5 min at 94°C, then 30 sec at 94°C, 45 sec at 48°C, 45 sec at 72°C, 30 times, and finally 5 min at 72°C. PCR products were denatured and run for 4 hr at 1700 V in a denaturing acrylamide gel (5% acrylamide) in 1XTBE buffer.

The second DNA sample was analyzed with a molecular method (M2) based on the amplification of a portion of an exon of the CHD1 gene. The detection of differences between males and females is achieved by a single-strand conformation polymorphism (SSCP) approach (Cortés et al. 1999) using the previously described primers P2 and P3 (Griffiths and Tiwari 1995). The PCR reactions contained about 50 ng of template DNA, 1X reaction buffer supplied by the manufacturer, 2 mM $MgCl_2$, 60 μM of each dNTP, 10 μM each primer, and 0.15 unit of *Taq* polymerase (Promega, Madison, WI U.S.A.). The initial denaturating period of 94°C for 2 min was followed by 35 cycles of 50 sec at 92°C, 1 min at 45°C, and 1 min at 72°C. The program was completed by a final step of 1 min at 72°C. All PCR reactions were performed in a Stratagene Robocycler. PCR products were denatured for 30 sec at 96°C and subsequently cooled to 0°C in ice. Sample electrophoresis was performed in a 14% nondenaturating polyacrylamide gel (29:1 Acrylamide/Bisacrylamide) in 1X TBE buffer at 14°C for 15 hr at 200V and DNA bands were visualized by silver staining.

Morphometric Measurements. The following measurements were taken following Ferrer and de le Court (1992) and Mañosa et al. (1995): body mass, measured with a dynamometer to the nearest 50 g; tarsus length, from the back of the tarsal joint to the front of the folded central toe; antero-posterior tarsus diameter at the middle point of the leg; culmen length from bill tip to the distal edge of the nostril; lengths of the hind and central fore claws, measured dorsally from the base to the tip of the claw; foot length, measured ventrally with the foot resting on a flat surface, from the base of the central fore claw to the base of the hind claw; and forewing length, from the front of the folded wrist to the proximal extremity of the ulna. These measurements were taken with calipers to the nearest 0.1 mm (1 mm in the case of the forewing length). The lengths of the stretched seventh primary and central tail feather were measured with a metal ruler to the nearest 1 mm from the tip of the feather to the skin insertion point, and they were used to estimate the age of nestlings (Mañosa et al. 1995). For a few birds only some of the body measurements were recorded.

Data Analysis. In the analysis we used the nestlings that were sexed by both molecular techniques (M1 and M2) and that showed consistent results among methods. When repeated biometric recordings were taken on the same individual over the breeding season, we only included in the analysis the last taken measurement. We discarded five birds that were only measured before they were 35 d old. This age was considered an adequate cutoff, because the size of most body structures tends to level off at about this age (Mañosa et al. 1995), and because handling of nestlings younger than this is uncommon in most field studies.

Univariate *F*-tests (Zar 1996) were used to test each measurement for significant differences between males and females. Significance of the tests was assessed after correction for multiple comparisons using the sequential Bonferroni technique (Rice 1989). Significant measurements ($P < 0.05$) were then used in linear discriminant analysis (Huberty 1994) to identify the variables or combinations of variables providing the maximum separation between sexes. Selection of variables was carried out with a forward stepwise procedure, but we forced the estimated age of nestlings (in d) in the equation to account for the eventual variation in body measurements due to growth. We used a cross-validation procedure to assess the predictive power of the discriminant functions, in which each individual was classified using a function derived from the total sample less the individual being classified (Huberty 1994). We also used the discriminant functions to corroborate gender for individuals sexed by only one of the two molecular techniques (external sample). All statistical analyses were conducted with SPSS v9.0 (SPSS Inc. 1998).

RESULTS

Molecular Sexing. The sexes of all four captive adult Bonelli's Eagles were correctly identified by