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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

Table 1. A comparison of means of external measurements between 35–50 d-old male and female nestling Bonelli's Eagles from southern Portugal (1994–99) sexed using molecular techniques. Measurements are given in g or mm. Asterisks represent significant differences for multiple comparisons based on sequential Bonferroni's correction for 10 simultaneous tests and table-wise error rate of 0.05.

	Females				Males					
Variables	*N*	Mean	SD	Range	*N*	Mean	SD	Range	*F*	*P*
Body mass[a]	25	2112	132	1850–2400	15	1660	97	1500–1800	132.96	<0.001*
Tarsus length	27	113.4	5.6	100.9–121.0	16	110.2	5.8	104.0–129.0	3.26	0.078
Tarsus diameter	27	12.6	1.5	10.6–17.6	16	11.3	1.4	9.0–14.6	8.09	0.007*
Fore claw length	27	27.1	1.4	22.1–29.6	16	26.0	1.6	24.7–30.6	5.28	0.027
Hind claw length	27	34.5	1.3	31.7–36.5	15	31.7	1.2	29.8–33.4	45.89	<0.001*
Foot length	19	125.3	5.1	111.5–135.3	12	116.8	4.5	111.4–129.0	22.23	<0.001*
Primary length	27	190.2	23.2	127.0–239.0	16	197.6	18.2	157.0–231.0	1.20	0.280
Tail length	26	141.0	18.3	102.6–167.0	16	146.9	13.8	121.0–165.0	1.25	0.271
Culmen length	27	25.2	1.3	21.8–28.2	16	23.6	0.9	22.0–25.1	19.32	<0.001*
Forewing length	25	188.0	8.9	159.5–210.0	16	177.9	4.8	169.0–185.0	17.22	<0.001*

[a] An exceedingly light female (1300 g) was considered an outlier due to disease and undernourishment, and thus it was excluded from this analysis.

both molecular sexing methods. The two adult females presented a heterozygous phenotype, corresponding to the amplification of a portion of the CHD1 gene from both the Z and W chromosomes, whereas the two males were homozygous, corresponding to the amplification of both copies from chromosome Z. For males, the M1 method yielded a single PCR product of about 380 bp as expected from the location of the primers used for PCR amplification, and the females presented an additional product of about 385 bp, suggesting that intron 2 of the W and Z chromosome differ by an insertion/deletion of a few base pairs. The M2 method yielded one PCR product of about 110 bp for both sexes, but females always displayed two bands while males showed a single band.

A total of 62 of 63 samples analyzed with M1, and 50 of 56 samples analyzed with M2, allowed a clear identification of sex for nestling Bonelli's Eagles. The only exception with M1 was a sample yielding a migration pattern of the PCR product totally different from the others, which was interpreted as the result of contamination. PCR amplification of six samples analyzed with M2 produced very few copies of the desired DNA fragment; those samples showed no bands in the SSCP gel and were therefore not sexed. From the 49 samples that could be sexed by both methods, results were congruent in all but one case (98.0%). This was a bird identified as female by M1 and male by M2.

Morphometric Analyses. Given the results of molecular sexing, 16 males and 27 females older than 35 d of age were available for morphometric analyses. In this sample, the mean age estimated for males ($\bar{x}$ = 45.6 ± 2.8 d, ± SD, range = 40–49 d) and females ($\bar{x}$ = 44.4 ± 3.6 d, range = 37–50 d) were similar ($F_{1,41}$ = 1.183, P = 0.283), thus any difference between sexes in mean body measurements could not be attributed to variation in age.

Except for an exceedingly light female (1300 g), the ranges of body mass for both sexes were nonoverlapping, with males averaging about 80% of the female weights (Table 1). The only female mismatch was considered an outlier and it was not used in further analysis involving weight, because it was a diseased and undernourished bird. Body mass alone provided a useful criteria for the determination of gender in Bonelli's Eagle nestlings, assigning to the correct sex about 92% of the weighed individuals, in both cross and external validation (Table 2). Although the diameter of the female tarsus and the lengths of the hind claw, foot, culmen, and forewing were also larger than that of males, these measurements presented considerable overlap between the sexes (Table 1), making them unreliable for sex determination (Table 2).

Body mass (BM) and hind claw length (HCL) were retained in the stepwise discriminant analysis together with age, which was forced into the equation. The resulting linear function (D_1 = 25.049 − 0.007BM − 0.702HCL + 0.283age) assigned all but

Table 2. Accuracy of sexing nestling Bonelli's Eagles (35–50 d), obtained from discriminant analysis using single measurements or linear combinations of morphometric variables, as assessed by cross-validation and by the classification of an external sample.

		Cases Correctly Separated							
		Cross-validation				External Sample			
		Females		Males		Females		Males	
Variable	Wilk's Lambda	%	N	%	N	%	N	%	N
Body mass	0.222	88.0	(25)	100.0	(15)	80.0	(5)	100.0	(8)
Tarsus diameter	0.835	59.3	(27)	75.0	(16)	100.0	(4)	87.5	(8)
Hind claw length	0.466	85.2	(27)	100.0	(15)	100.0	(4)	100.0	(8)
Foot length	0.566	78.9	(19)	91.7	(12)	100.0	(4)	100.0	(5)
Culmen length	0.680	74.1	(27)	81.3	(16)	100.0	(4)	87.5	(8)
Forewing length	0.694	72.0	(25)	81.3	(16)	50.0	(4)	100.0	(6)
Linear discriminant functions									
Function D_1	0.122	92.0	(25)	100.0	(14)	100.0	(4)	100.0	(7)
Function D_2	0.253	96.3	(27)	100.0	(15)	100.0	(4)	87.5	(8)

Table 3. Comparisons of the frequency distributions of discriminant scores between male and female nestling Bonelli's Eagles (35–50 d), from southern Portugal (1994–99), along the axis defined by the discriminant functions D_1 and D_2.

Discriminant Scores	No. of Females	%	No. of Males	%
D1				
<−3.0	6	24.0	0	0.0
]−3.0, −2.0]	8	32.0	0	0.0
]−2.0, −1.0]	5	20.0	0	0.0
]−1.0, 0.0]	4	16.0	0	0.0
]0.0, 1.0]	2	8.0	0	0.0
]1.0, 2.0]	0	0.0	0	0.0
]2.0, 3.0]	0	0.0	3	21.4
]3.0, 4.0]	0	0.0	8	57.1
>4.0	0	0.0	3	21.4
Total	25		14	
D2				
<−2.0	7	25.9	0	0.0
]−2.0, −1.0]	7	25.9	0	0.0
]−1.0, 0.0]	11	40.7	0	0.0
]0.0, 1.0]	1	3.7	2	13.3
]1.0, 2.0]	1	3.7	6	40.0
]2.0, 3.0]	0	0.0	3	20.0
>3.0	0	0.0	4	26.7
Total	27		15	

two individuals to the correct sex (overall success = 96%), where values of $D_1 > 0$ identified males and values < 0 identified females (Table 2). In some circumstances data on body mass may not be available (e.g., carcasses); therefore, we repeated the stepwise discriminant analysis excluding this variable. The resulting discriminant function ($D_2 = 25.624 - 1.072HCL - 0.239DT + 0.295age$) included claw length, tarsus diameter (DT), and age and it classified all but one bird correctly in both cross- and external-validation (overall success = 96%). The frequency distribution of discriminant scores indicated that in most cases males were well separated from females by the linear combinations of variables D_1, although not as well by D_2 (Table 3). The separation was much smaller when the effects of age were not accounted for, particularly in the case of D_2. The single sample for which there was disagreement among molecular techniques was clearly classified as a female irrespective of the morphometric criterion used, thus supporting the results of M1.

Discussion

Molecular Sexing. The lower success in sexing nestlings with M2 (one putative error and six blanks) than with M1 (one blank) could be partially attributed to the lower quality of the samples used, which had been frozen and thawed several times before DNA extraction and amplification. If good-quality samples were used, a higher success rate could have been achieved, probably similar to

that obtained by M1. However, the difference in success rate between the two methods may also have resulted from the techniques themselves, thus indicating that M1 may be more robust than M2. For one sample, the M2 method apparently gave a false result (a male instead of a female pattern). One explanation for this could be that the M2 method is expected to allow the detection of single point mutations, while M1 can only detect differences in length of about 5–10 bp between homologous fragments. This difference in sensitivity to detect sequence variation could explain the difficulty in the interpretation of results and the discrepancy between methods, as M2 would produce an unknown migration profile for each variant, including potential false female or false male patterns. Therefore, M2 is apparently less robust than M1 because of the lower success rate of interpretable migration profiles and probably is also less reliable than M1 according to the false result obtained. An important factor is the amount of effort (quantity of products, money, and time) necessary for each method. The results can be obtained within 24 hr using M2 and 48 hr with M1, and the cost of the products is higher for the latter method (e.g., radioactive labeling, large sequencing gel, and autoradiography exposure). Thus, M2 is globally quicker and less costly but also less efficient. This balance strengthens the need for a simple morphological way to determine the sex of Bonelli's Eagle nestlings.

Morphometrics. Our results clearly demonstrated a marked sexual size dimorphism for most external body measurements in Bonelli's Eagle nestlings from age 35–50 d. The main exceptions were the lengths of the seventh primary and the central tail feather, which were remarkably similar between males and females at any given age. These two measurements have been used in the age estimation of nestlings (Mañosa et al. 1995) on the assumption that feather growth shows small variations between sexes, as is typical of other raptor species (Poole 1989, Sodhi 1992). Although this issue was not addressed directly in this study, our results do support this assumption, and thus the aging method proposed by Mañosa et al. (1995).

Body mass, either alone or combined with other variables, provided the most consistent cue for sexing the nestlings. However, the use of this parameter should be regarded with some caution, for mass is highly variable, even within a 24-hr period, and depends on growth rate, degree of hydration, amount and time of the most recent meal, among other factors. Nevertheless, our results suggest that the differences in mass between sexes tend to prevail over the background of natural variability that may be present. There was a single individual that could not be classified by any discriminant function including mass, and this was an extremely lightweighted female, with a severe infection caused by the protozoan parasite *Trichomonas gallinae* (trichomoniasis). This disease induces large, fibrous lesions in the oesophagus and oropharynx preventing birds from swallowing food, and eventually leading to death by starvation (Höfle et al. 2000). Care should thus be taken when sexing nestlings on body mass criteria, if signs of severe trichomoniasis and emaciation are apparent. This same bird could, however, be sexed with the linear discriminant function D_2, suggesting that even diseased birds can be sexed on the basis of morphometric criteria. In these circumstances, however, molecular sexing techniques are likely to provide more reliable results.

The estimated age of nestlings was included in both discriminant functions, though this variable did not improve the correct assignment of sex of the sampled individuals. However, when age was forced into the equations, the separation between groups along the discriminant axis was always increased (expressed by the Wilk's lambda). Although the growth of most body structures levels off at about 35 d, they tend to continue growing at slow rates almost until fledging (Mañosa et al. 1995), making the difference between the two sexes more evident when nestlings of the same estimated ages are compared. Therefore, by considering the age of nestlings, we achieved more robust discriminant functions, providing more confidence to the classifications obtained with these models.

In conclusion, the results of this study demonstrated that external morphometry may be used for the determination of sex in nestling Bonelli's Eagles from 35–50 d. The discriminant measurements needed to use our method are easy to obtain in the field, allowing an immediate and about 96% accurate determination of sex. Difficulties may arise, however, in the case of undernourished or diseased birds, for which our equation D_1 should not be used. In general, we recommend that both discriminant equations should be computed for each bird to assess the internal coherence of the sexing results. Whenever possible and logistically feasible, molecular sexing should be used along

with morphometrics to reduce the overall error rates, particularly in those instances where it is essential to know, with absolute precision, the sex of every individual handled.

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