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An approach to sexing young Great Bustards *Otis tarda* using discriminant analysis and molecular techniques

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Adult Great Bustards Otis tarda *are sexually dimorphic, males weighing more than twice as much as females. However, there is no practical way to distinguish sex in chicks by their morphology. In this paper we describe a discriminant function, Tail Length/Weight, which correctly sexed 98.2% of 165 Great Bustard chicks at two study areas in Spain, the Wildlife Reserve of Lagunas de Villafáfila and the province of Madrid. The value for Tail Length/Weight separating the sexes was 0.099: Tail Length/Weight for males < 0.099 < Tail Length/Weight for females. We also show that the recently described PCR-based sex determination technique using genomic DNA is valid for the Great Bustard. Both approaches should be useful for sexing young Great Bustards in captive breeding programmes and studies on wild populations.*

In sexually monomorphic birds adults cannot be sexed by their external appearance, and very few species, including dimorphic ones, can be sexed as chicks. Recently several methods have been developed for the sex determination of birds, based on either discriminant analyses using body measurements or molecular analyses of genetic material. Discriminant analysis based on morphological measurements has been successfully used to determine the sex in adults of several species of birds with monomorphic plumage.[1–7] The need for a morphometric discriminant technique is less evident for birds that show sex dimorphism in size as adults. However, even in these species it is still useful if there is overlap between sexes in linear measurements,[8,9] or in determining the sex of juveniles or immatures, when size or plumage dimorphism is often not completely developed. Sex determination of chicks or juvenile birds is important for studying differential mortalities of male and female offspring and evaluating current sex ratio theories.[10–12] It may also be useful in captive breeding and reintroduction programmes of endangered species.

The Great Bustard *Otis tarda* is a globally endangered species[13] in which numerous populations are now extinct. Consequently, long-term intensive captive breeding programmes, aimed at saving or reintroducing the species, have been operating in Germany[14] and Hungary.[15] Sexual dimorphism in size develops in Great Bustards at a relatively early age: captive-bred males weigh more than females at three weeks old, and almost twice as much when they are three months old.[16–18] However, no single morphological measurement or plumage characteristic is known that permits reliable sexing at this early age.

Here we describe a discriminant function that differentiates the sex of Great Bustards at an age of only a few weeks. In addition, we present the nucleotide sequence of a part of the Z- and W-linked CHD gene for accurate molecular sexing using a polymerase chain reaction (PCR) on genomic DNA, following the

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technique described by Griffiths *et al.*[19] and Ellegren.[20]

A final application of sexing techniques of young Great Bustards is to choose the most appropriate marking system, as some methods are not valid for males owing to their much greater growth rates. For example, young Great Bustards of unknown sex have sometimes been banded with metal rings and later found dead after suffering from severe limping due to constriction of tibia or tarsus by a ring that was too small (pers. obs.). Also, some radio-tagging methods such as neck-collars, neck-laces or ponchos[21] can only be fitted to female chicks, because in adult males they would prevent normal inflation of the air sacs during sexual display.

METHODS

Study populations and data collection

We captured 271 young birds at two different study areas. The first site was the Wildlife Reserve of Lagunas de Villafáfila, NW Spain, which holds the world's densest population of the species.[22,23] The second site was in the province of Madrid, central Spain, where several breeding groups are endangered by habitat fragmentation. All chicks (164 during 1987–93 at Villafáfila, 107 during 1995–97 at Madrid) were captured at ages of 20–70 days, while they are still dependent on their mothers, and released within 30 minutes after marking (respectively 63 birds with wingtags and 208 with wingtags and radio-transmitters). In all cases we confirmed that the chicks released after marking were soon rejoined by their mothers.

The following measurements were taken from each bird. *Maximum Wing Length* or *Wing Arch*: maximum distance between the carpal joint and the tip of the longest primary, measured with a tape along the dorsal side of the wing. *Unflattened Wing Length* or *Wing Chord*: minimum distance between the carpal joint and the tip of the longest primary feather. *Tail Length*: length of the longest tail feather pushing the bottom of the ruler gently against the base of the middle pair of tail feathers while the tail is folded naturally. *Tarsus Length*: distance between the notch on the back of the intertarsal joint and the lower edge of the last complete scale before the toes diverge. *Central Toe Length 1*: distance between lower end of tarsus and central toe tip excluding the claw, with the toe stretched. *Central Toe Length 2*: holding the tarsus and central toe at a right angle, the total length of the stretched toe, excluding the claw, measured by pushing the basal end of the toe against one end of the calliper (this measurement is easier to take than the previous one, because it is not necessary to find the joint between tarsus and central toe, Fig. 1). *Head Length*: maximum distance between the occipital end of the head and the tip of the bill. *Head Width*: maximum width of the skull behind the eyes. *Bill Length 1*: distance between the rear end of bill commissure and bill tip. *Bill Length 2*: distance between the rear end of nostrils and bill tip. *Weight* (in g). All measurements were taken by the same person, using the same measuring tools, and to the nearest 1 mm (Wing Arch, Wing Chord and Tail Length), 0.1 mm (the rest of linear measurements) or 50 g interval.

Because all birds were individually marked, their sex could be determined in the field several weeks after marking, during the maternal dependence period by comparison with the size of the mother, and confirmed later during the immature or adult periods.[24,25] At an age of about four months, male chicks have reached the size of their mothers, while female chicks are still smaller.

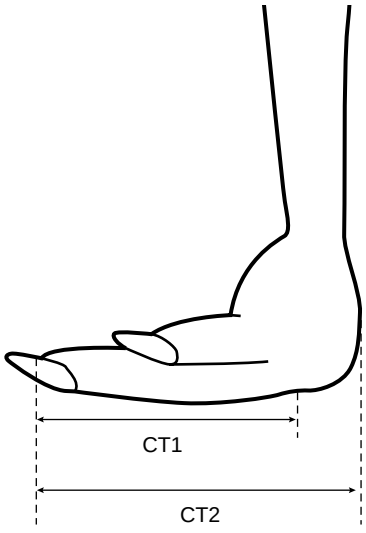

Figure 1. Measurements of central toe length used in this study: CT1 = Central Toe 1; CT2 = Central Toe 2.

Owing to the high juvenile mortality which occurs naturally during the first months after hatching,[26] many birds from the initial sample could not be sexed and so were excluded from the analysis. The final sample was 92 males and 73 females.

Data analysis

Stepwise discriminant analysis (SDA; STATISTICA 4.5 program[27]) was used to separate young Great Bustards by sex. At each step this method selects the variable most useful in differentiating the groups: an F statistic based on the one-way test of analysis of variance is used in the selection. Values of $F = 4$ and $F = 3.9$ were used to enter and remove variables in a forward manner. The assignment of cases to the groups was carried out by using the classification functions (one for each group) calculated from the selected variables.[28,29]

Analysis of the blood samples and molecular sexing

Blood samples were used to develop a molecular sexing process. Using a syringe we withdrew 0.4 ml of blood from the brachial veins of 107 birds and stored the samples in Queen's buffer[30] at –70°C. Genomic DNA was extracted from 20 individuals selected at random from the total sample, using proteinase K digestion followed by standard phenol/chloroform extraction.[31] Blood samples were homogenized in 100 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 mM NaCl, 0.1% SDS, and 10 μg ml^{-1} proteinase K, and incubated overnight at 55°C. The homogenate was extracted twice with Tris-saturated phenol and once with chloroform-isoamyl alcohol (24:1). Finally, nucleic acids were precipitated with sodium acetate and ethanol, and resuspended in distilled water.

Using the polymerase chain reaction (PCR[32]) with primers P_3 and P_2 (AGATATTCCGGATCTGATAGTGA and TTTCCTAAATCGCTACGTCT), both copies of the CHD gene in Great Bustards were amplified following the protocol of Griffiths *et al.*[19, a]

RESULTS

Discriminant analysis

Males were significantly larger than females in all morphometric variables, although there was considerable overlap between sexes (Table 1). The best bivariate discriminant function, obtained by subtraction of the two corresponding classification functions,[28] included the Central Toe Length 2 and the Tail Length, and correctly classified 96.5% of individuals:

$$D_{MF} = 1.0818\ \text{Central Toe Length 2} - 0.0983\ \text{Tail Length} - 45.8475 \quad [1]$$

Using this formula, a bird would be male if $D_{MF} > 0$ and female if $D_{MF} < 0$.

Most of the overlap between sexes was due

Table 1. Sex differences in various measurements of young Great Bustards.

	Males				Females				
	Mean	sd	min–max	*n*	Mean	sd	min–max	*n*	*t*
Wing Arch	450.5	50.5	295–550	89	382.4	35.4	275–460	72	10.0**
Wing Chord	403.3	43.3	270–500	89	348.4	33.1	260–445	72	9.1**
Tail Length	199.3	26.9	110–250	92	179.3	23.3	104–250	72	5.0**
Tarsus Length	125.5	12.1	91.5–149.8	92	108.2	9.9	85.7–135.0	73	10.0**
Central Toe L.1	60.2	5.0	50.3–73.5	90	49.0	3.4	39.2–57.0	73	16.7**
Central Toe L.2	66.0	4.9	55.2–84.5	79	53.4	3.0	44.6–59.5	63	18.5**
Head Length	115.7	7.0	94.5–131.7	91	100.4	5.1	87.0–111.0	73	16.0**
Head Width	42.6	2.5	34.0–48.5	90	37.6	1.7	31.9–40.5	73	14.3**
Bill Length 1	67.7	4.9	57.0–80.7	89	58.1	4.1	46.3–72.0	73	13.4**
Bill Length 2	26.1	5.4	18.8–40.0	91	21.8	2.8	17.2–31.6	72	6.3**
Weight	2494	483	1250–3700	92	1562	276	950–2300	73	15.5**

All linear measurements in mm, weight in g. Student's *t*-test: $^{**}P < 0.01$

Table 2. Sex differences in linear measurements divided by weight in young Great Bustards.

	Males				Females				
	Mean	sd	min–max	*n*	Mean	sd	min–max	*n*	*t*
Wing Arch/Weight	0.183	0.022	0.141–0.236	89	0.249	0.031	0.189–0.358	72	15.0**
Wing Chord/Weight	0.164	0.020	0.122–0.216	89	0.228	0.029	0.170–0.326	72	15.7**
Tail Length/Weight	0.081	0.094	0.065–0.107	92	0.117	0.012	0.100–0.159	72	20.0**
Tarsus Length/Weight	0.051	0.006	0.039–0.073	92	0.071	0.009	0.056–0.096	73	15.0**
Central Toe L1/Weight	0.025	0.003	0.018–0.040	90	0.032	0.004	0.024–0.042	73	11.2**
Central Toe L2/Weight	0.027	0.003	0.020–0.037	79	0.036	0.005	0.027–0.048	63	11.7**
Head Length/Weight	0.048	0.007	0.036–0.076	91	0.066	0.009	0.048–0.095	73	13.2**
Head Width/Weight	0.018	0.002	0.013–0.029	90	0.025	0.003	0.017–0.037	73	13.2**
Bill Length 1/Weight	0.028	0.004	0.021–0.046	89	0.038	0.005	0.030–0.054	73	13.5**
Bill Length 2/Weight	0.011	0.002	0.007–0.019	91	0.014	0.003	0.010–0.026	72	8.9**

Student's *t*-test: $^{**}P < 0.01$

to the inclusion in our sample of birds of different ages, and thus quite variable degrees of body growth. In order to minimize such overlap we divided all single linear measurements by the Weight, as a way to compensate for age differences, since age and weight are related linearly at this age.[16] Dividing by Weight resulted in a marked increase in sex differences when using Wing, Tail, Tarsus and Bill Length (see *t*-values in Table 2), as it contributed to the separation of the more bulky male chicks from the more slender females. In this way we expected a notable increase in the discriminating power of the analysis. We performed a new SDA with all 21 variables, including the ten linear measurements, Weight, and the ten linear measurements divided by Weight. The variable selected in the first step was Tail Length/Weight, with a correct classification of 98.2%. The discriminant function using only this variable was:

$$D_{MF} = -320.8704\,(\text{Tail Length}/\text{Weight}) + 31.945 \quad [2]$$

Again, this formula describes males if $D_{MF} > 0$ and females if $D_{MF} < 0$.

Discriminant function 2 classified correctly more birds than function 1, using only one linear measurement instead of two. With function 2 only three males out of 165 birds were incorrectly classified as females. Adding new variables by allowing for more steps in the analysis resulted in 100% correct classification of cases:

$$D_{MF} = -607.703\,(\text{Tail Length}/\text{Weight}) + 0.666\,\text{Central Toe Length 2} + 185.892\,(\text{Wing Arch}/\text{Weight}) - 19.932 \quad [3]$$

However, the difference in discrimination power between functions 2 and 3 probably does not justify measuring Central Toe and Wing Arch in the field.

To facilitate a rapid and practical sexing in the field, we calculated the midpoint between male and female sample means for Tail Length/Weight. The value obtained was 0.099, which can thus be used with 98.2% confidence as a limit between males and females: Tail Length/Weight for males < 0.099 < Tail Length/Weight for females.

Finally, we performed SDA separately for each of the two study areas. In both analyses the variable entered in the first step was Tail Length/Weight, the coefficients of the discriminating function were very similar and the sex determination success rate and the limits between male and female samples almost identical (respectively, 96.9% and 0.096 for Villafáfila; 97.0% and 0.103 for Madrid).

Molecular sexing

A comparison between the two avian CHD genes by nucleotide sequence analysis showed that the Z-linked CHD fragment contains a unique *HaeIII* site in the Great Bustard (Fig. 2). This allows easy distinction between males and females in this species. Nine of the 20 blood

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Gallus CHD-W AGA TGT TCT GGA TCT GAT AGT GAT TCC ATC TCA GGA AGA AAA CGA CCA
Otis   CHD-W ... ... ... ... ... ... ... ..C ... ... ... ... ... ... ..T
Gallus CHD-Z ... ... ... ... ... ... ... ..C ... ... A.. ... ... ... ..G ...
Otis   CHD-Z ... ... ..C ... ... ... ... ..C ... ... ... ... ... ... ..G ...
                     *                                 DdeI          HaeIII*

Gallus CHD-W AAA AAA CGT GGA CGA CCA CGA ACT ATT CCC CGT GAA AAC ATT AAA GGA
Otis   CHD-W ... ... ... ... ... ... ... ... ... ..T ..A ... ..T ... ... ...
Gallus CHD-Z ... ..G ... ... A.. ..T ... ..C ... ..T ..A ... ..T ... ... ...
Otis   CHD-Z ... ... ... ... A.. ... ... ..C ... ..T ..A ... ..T ... ... ...

Gallus CHD-W TTT AGT GAT GCA GA
Otis   CHD-W ... ..C ... ... ..
Gallus CHD-Z ... ... ... ... ..
Otis   CHD-Z ... ..C ... ... ..
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Figure 2. Partial nucleotide sequences of both W- and Z-linked CHD genes in the Domestic Fowl and Great Bustard, all arranged as putative codons. Dots denote nucleotides shared with the homologous CHD-W gene from the Domestic Fowl. Stars under the aligned sequences indicate synonymous mutations in the Great Bustard. The *DdeI* (CTNAG) and *HaeIII* (GGCC) sites are underlined.

samples were males. Testing the discriminant functions 2 and 3 on this sample of 20 birds with DNA-verified sex resulted in 100% accurate classification.

The primers P_2 and P_3 amplified a CHD Z-derived fragment of 110 bp in both sexes. The same PCR also amplified the W-linked CHD fragment only in female birds. The 110 bp CHD-Z male PCR fragment was cut by *HaeIII* restriction enzyme into two fragments (45 bp, 65 bp) , while in females CHD-W was not cut by *HaeIII* and remained at 110 bp.

DISCUSSION

Although size dimorphism in adult Great Bustards is one of the highest among birds, young of this species cannot be accurately sexed by plumage characteristics or any single external measurement during their first months of life.[18,33] We present two ways to sex young Great Bustards: a discriminant analysis using body measurements and a molecular technique. In the sample used for the discriminant analysis, we found significant differences between sexes in their mean body measurements, but the substantial overlap in all of them prevented the use of a single measure to accurately sex birds. The high overlap between sexes was probably due to the presence in the sample of birds of quite different ages, and therefore various degrees of body growth. Age is usually unknown in studies of free-living altricial species where the nest is difficult to find and thus the hatching date difficult to determine. To compensate for age differences between individuals, we divided all linear measurements by Weight, and obtained two discriminant functions. The first classified correctly 98.2% of the birds using only Tail Length and Weight. Thus, this function was simple, powerful and easy to apply in the field. The second function sexed accurately 100% of the birds using two additional body measurements, the Central Toe Length 2 and the Wing Arch.

The three males incorrectly classified as females by the discriminant function using only Tail Length and Weight were probably undernourished individuals, with lower than normal weights for their skeletal and feather development. The slimness of one of them was already apparent while handling it during measuring and tagging. Another misclassified male weighed 2000 g but had a sibling that weighed 2900 g, which also suggested that the weight of the first bird was abnormally low for that age (about 40–50 days). Undernourishment is one of the common causes of juvenile mortality in this species, affecting particularly male offspring because of their much faster body growth and higher nutritional requirements which peak at an age of between 2 and 4 months.[26,34]

In addition to the discriminant analysis, we present the results of applying the method described by Griffiths *et al*.[19] and Ellergen[20] to the Great Bustard. These authors developed a sexing technique based on the W-linked CHD gene, and tested it in several bird species, suggesting that this method was likely to provide a universal sexing system for birds other than ratites. Our study shows the validity of this molecular test for a species of the family Otididae, supporting its general applicability to the avian class.

The discriminant function developed in this paper has several applications as an easy, rapid technique to sex young during both captive breeding programmes and in behavioural studies in the wild. Accurate sexing of young Great Bustards caught for individual marking permits correct choices on the appropriate banding or tagging method and, in particular, averts the accidental use of small rings or neck-collar-mounted radio-transmitters in males. The discriminant function also represents a quick and reliable method to determine sex ratios of Great Bustard offspring at least from 3 to 10 weeks, while the molecular technique enables sexing at any age, including eggs and recently hatched chicks, as well as remains of freshly dead birds. The potential use of these techniques when evaluating current sex ratio theories[10–12] and studying early juvenile mortality is evident. The discriminant method proposed in this study has already proved to be useful in determining the sex of young of at least one other species of bustard, the Kori Bustard *Ardeotis kori* (T. Osborne & L. Osborne, unpubl. data), and may have utility for other bird species which develop sex dimorphism in size as adults.

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ENDNOTE

a. Because the PCR can amplify particular DNA segments from tiny amounts of starting tissue, it has extended molecular applications to a much wider biological arena. The thermal profile was as follows: 94°C for 3 min; 30 cycles of 15 s at 94°C, 20 s at 57°C, 45 s at 72°C, and finally 7 min annealing and extension steps at 57°C and 72°C respectively. The PCR products were purified (QIAquick PCR purification kit/Qiagen), sequenced by the extension-dideoxy-chain termination method[35] with a commercial kit (Dye Terminator Cycle Sequencing Kit, Applied Biosystems/Perkin Elmer), and analysed on an ABI373 Sequencer (Applied Biosystems). The sequence information obtained was used to identify discriminatory restriction endonuclease sites. *HaeIII* (five units; Boehringer Mannheim) was used to cut 8 μl of PCR product in 1× restriction enzyme buffer M (Boehringer Mannheim) and 50 ng μl^{-1} spermidine in a total volume of 10 μl. The digested and uncut PCR products were precipitated before undergoing electrophoresis in a visigel (Stratagene) at 3.5 V cm^{-1} and later visualized using ethidium bromide (40 ng ml^{-1}).

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