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Sexing of Black-tailed Godwits *Limosa limosa islandica*: a comparison of behavioural, molecular, biometric and field-based techniques

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Capsule Biometrics and plumage characteristics can both be used to reliably sex Black-tailed Godwits.
Aims To develop methods of sexing Black-tailed Godwits and to validate their relative accuracy.
Methods A sample of 84 Black-tailed Godwits was sexed by DNA analysis of feather samples. The biometric data and plumage characteristics of these birds were then used to develop protocols for sexing godwits in the field.
Results A discriminant function analysis of biometric data correctly sexed 95% of the DNA-sexed reference sample. Of 808 birds caught throughout the range, 74% could be sexed with this method. Approximately 85% of the reference sample were correctly sexed on three plumage characteristics in the hand. Of 105 birds sexed by DNA or biometrics, 82% were sexed correctly on general impression and shape in the field.
Conclusions For the many species with limited sexual dimorphism, a relatively small sample of accurately sexed birds can provide a means of testing and improving current morphological methods of sexing.

Many studies of a wide range of species have shown that important ecological processes can vary between the sexes. For example habitat use and diet frequently vary (Anderson & Norberg 1981, Shine 1991, Durell & Atkinson 2004) and so do the resulting behavioural and physiological processes (Durell 2000, Tschirren *et al.* 2003). Any studies of sexual differences in behaviour, ecology or evolution require reliable means of sexing individuals. Some species show extreme sexual dimorphism, such as Peacocks *Pavo muticus*, and sexing is then straightforward. However, many species are monomorphic or show little dimorphism. For example, of 108 species of shorebirds (Charadrii), 82% are weakly dimorphic in either or both of size or plumage characteristics, whereas only 9% are clearly dimorphic (Prater *et al.* 1977). This inability to distinguish the sexes is considered to be an important reason why some aspects of sex differences in ecological processes, such as differential migration or feeding specializations, are poorly understood in shorebirds (Durell 2000). In truly monomorphic species, sexing may only be possible using methods such as molecular techniques, many of which require tissue samples. However, in those species where some dimorphism exists, field-based and laboratory methods may complement each other (Martin *et al.* 2000, Devlin *et al.* 2004, Sarasola & Negro 2004), allowing researchers to test methods in order to produce sexing protocols that are both efficient and non-invasive.

Here we explore means of sexing Black-tailed Godwits *Limosa limosa islandica*, a weakly dimorphic species. On average, females are larger and their breeding plumage is paler; however, there is overlap in both biometrics and plumage traits. These overlaps are typical of most shorebird species (Prater *et al.* 1977) and such subtle sexual dimorphism is common amongst

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birds in general. In this paper we compare molecular methods with those based on biometrics and plumage characteristics, and we also use field trials to investigate feasible means of non-invasive sexing in this species.

METHODS

Icelandic Black-tailed Godwits were caught by cannon-netting, mist-netting and nest-trapping in Iceland, the UK and France. In Iceland, catches were made on six spring staging sites around the country, and breeding birds were trapped while incubating. In the UK, catches of birds in autumn and winter have been made on the Solent in south England and on the Wash estuary in eastern England. In France, birds were caught in autumn in the Golfe du Morbihan in Brittany. A range of biometric data were recorded for these birds and all were marked with a metal ring and individual combinations of four colour-rings. In total, 1369 adult birds were caught and marked, with full biometric data collected for 808 of these. Birds were weighed to the nearest gram using Pesola balances. The chord of the exposed culmen (bill length), wing length (maximum chord) and tarsus length were measured to the nearest 0.1 mm with calipers. Juveniles were excluded from all analyses as most do not attain full size until their second year of life. Feathers were plucked from 84 of the birds, and DNA was extracted from the feathers for molecular analysis.

Sexing with molecular methods

Approximately 5 mm^3 of feather was taken from the proximal end of each feather shaft. The tissue was finely sliced and DNA was extracted using the QIAGEN DNEasy column kit after overnight digestion with Proteinase K. DNA was eluted in 100 μl water.

Two separate sets of primers were used for sexing the birds by PCR:

Set 1: P2, 5'-TCTGCATCGCTAAATCCTTT-3' with P8, 5'-CTCCCAAGGATGAGRAAYTG-3' (Griffiths *et al.* 1998); and

Set 2: 2550F, 5'-GTTACTGATTCGTCTACG-AGA-3' with 2718R, 5'-ATTGAAATGATCCAG TGCTTG-3' (Fridolfsson & Ellegren 1999).

Each set of primers amplifies an intron within the CHD1 gene, which is found on both of the Z and W sex-determining chromosomes in birds. The size of the intron amplified in each case differs between the two chromosomes and this allows us to distinguish between ZZ (male) individuals and ZW (female) individuals. Amplification of both sexes produces a band corresponding to the size of the intron on the Z chromosome, but in females there is an additional, larger band that corresponds to the size of the intron on the W chromosome.

Polymerase chain reactions were carried out in a total volume of 25 μl containing 1 unit of Taq (Abgene), 2.5 mM $MgCl_2$, 0.5 mM of each dNTP, 400 nM of each primer and 1 μl of DNA solution, in a buffer of 10 mM Tris-HCl, 500 mM KCl, pH 8.3 (20°C). An initial denaturation at 94°C for 1 minute was followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 20 seconds and extension at 72°C for 30 seconds. PCR products were visualized on a 3% agarose gel.

Sexing with copulation behaviour

From the individually marked breeding birds, observations of copulation behaviour were used to assign sex to 15 individuals (7 males and 8 females). We assumed that all these birds were heterosexual and adopted the appropriate copulatory position for their sex (i.e. males on top). The sex of these individuals was then compared to the sex assigned by the molecular sexing technique.

Sexing with biometrics

Using the 84 birds sexed by DNA as a reference sample, discriminant function analysis (DFA) provided a model for sexing Icelandic Black-tailed Godwits on biometrics. Wing and bill lengths were used as these measurements are standard, were available for most of the birds, and do not fluctuate as much as mass. Sex was assigned to those birds with probabilities of 95% or more of belonging to a particular sex. In order to explore the success of this method in predicting sex, the discriminant function analysis was initially carried out on a randomly selected half of the birds of known sex (n = 42). The resulting function was then used to predict the sex of the remaining 42 birds of known sex. Following this test, the discriminant function derived from the full data set of known-sex birds (n = 84) was used to predict the sex of the full sample of 808 individuals for which biometrics were available.

Sexing with plumage traits

During the breeding seasons of 2001–03, breeding Black-tailed Godwits were captured in Iceland and

Table 1. Definitions of the Black-tailed Godwit plumage parameters that were recorded during capture and handling.

Variable	Description	Categories
Head colour	Darkness of stripes on top of the head	Black or Grey
Breast redness	Intensity of red colour on the breast	Dark, Medium or Pale
Grey in back	Amount of grey in the back, from base of neck to upper part of rump	% grey feathers
Black in back	Amount of black in the back, from base of neck to upper part of rump	% black feathers
Barring definition	Definition of the barred area on the lower breast and belly	Strong, Medium or Diffuse
Eye stripe	Presence or absence of eye stripe	Eyestripe or no eyestripe

several plumage parameters were recorded during careful examination in the hand. As the aim was to investigate whether birds could be sexed in the field using plumage traits, we only explored the variables that could readily be recorded from a distance (Table 1). These measurements were available for 38 males and 39 females which had also been sexed using the molecular techniques. All the variables in Table 1 were entered into a logistic regression and removed one by one until only those that contributed significantly to the fit remained in the model.

Sexing in the field

Individually colour-ringed godwits, observed at distances of approximately 30–100 m with the aid of a telescope, were assigned a sex based on the observer's impression of size, particularly the proportional bill length, and plumage characteristics. Sex was assigned to all marked birds within adequate view. These trials were carried out on spring staging sites in Iceland in April and May 2002 and 2003. At this time birds are in full and fresh summer plumage. Field-sexing was then compared with sexing using both molecular methods and biometrics (at the 95% level). In total, sex was assigned to 105 birds in the field by two different observers whose success rates were compared.

RESULTS

Sexing with molecular methods

For the 15 birds that were sexed by copulatory behaviour, the sex assigned by molecular analyses confirmed that assigned by behaviour. Of the 84 birds that were sexed by DNA analysis, 52 were males and 32 females. Both sets of primers gave entirely consistent results and each PCR was performed at least twice and found to be 100% repeatable. Individuals found to be female by the DNA analysis had significantly larger bill and wing lengths (Table 2). However, there was an overlap in both measurements between the sexes (Fig. 1).

Sexing with biometrics

A discriminant function analysis incorporating wing length and bill length, for a randomly selected half (n = 42) of all individuals sexed by DNA, yielded a model which correctly predicted sex in 95.2% of the remaining 42 individuals of known sex, with all 26 males and 14 of the 16 females being correctly classified.

The discriminant function model incorporating wing length and bill length for all 84 individuals sexed by molecular analysis correctly classified 82 (98%) individuals (Fig. 2). The two misclassified cases were both females that, as in the previous analysis, were incorrectly sexed as males on the basis of their biometrics. This model yielded the following formula:

$$\text{Discriminant coefficient} = (\text{wing} \times 0.128678) + (\text{bill} \times 0.137847) - 40.0141$$

where wing is wing length (mm) and bill is bill length (mm). The discriminant coefficient yields a critical value for females of 1.21; any individual with a coefficient greater than or equal to this has a 95% or greater chance of being female. Similarly, the critical value for a male at the 95% level is –0.28 or smaller. The discriminant function analysis classified 74% of 808 godwits at the 95% level.

Table 2. Comparison of the mean (± sd) wing length and bill length of male and female Black-tailed Godwits that were sexed using molecular techniques.

	Males	Females	t
Wing length (mm)	214.3 (±4.16)	228.5 (±5.13)	14.19**
Bill length (mm)	79.3 (±3.46)	94.7 (±6.21)	13.23**
n	52	32	

**$P < 0.001$.

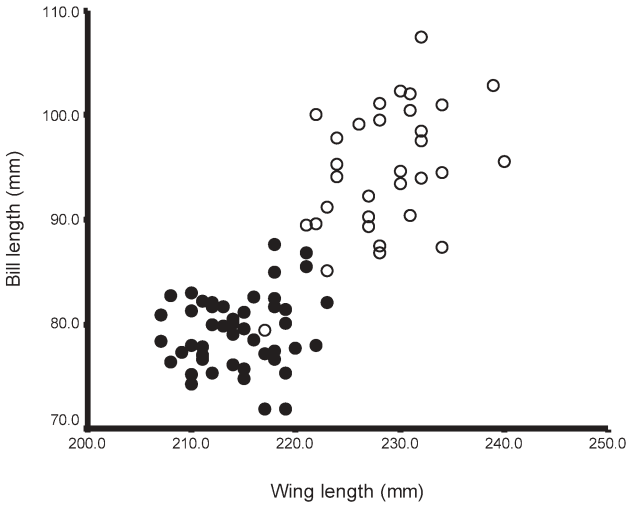

Figure 1. Bill and wing lengths of 84 Black-tailed Godwits of known sex (sexed by DNA analysis): ●, males; ○, females

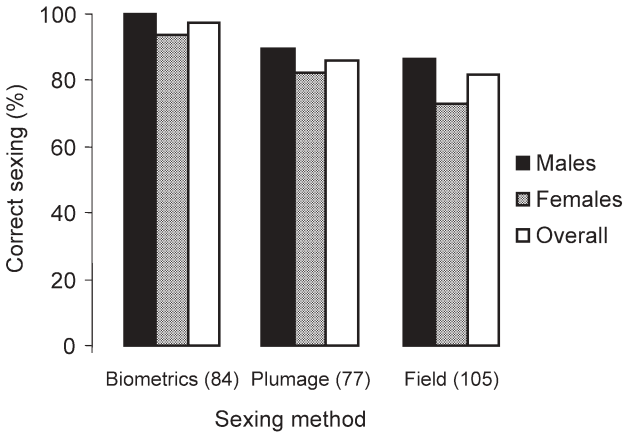

Figure 2. Accuracy of different methods of sexing Icelandic Black-tailed Godwits. Only birds sexed by DNA analysis were included in the discriminant function analysis of biometrics. Birds sexed by both DNA and biometric analyses were used to compare plumage and field techniques. See text for details. Sample sizes are given in parentheses.

Sexing with plumage traits

Of the six plumage traits that can be recorded in the field, three were retained in the final logistic regression model: grey in back (%), breast redness and colour of head stripes (Table 3). At the 95% cut-off point, the model classified 89.5% of males correctly and 82% of females correctly (Fig. 2). All three plumage variables differed significantly between males and females (Table 4). On average, males had *c.* 7% grey in the back whereas females had *c.* 25%. Males were more likely to have black stripes on head and darker red feathers on the breast and neck (Table 4).

Field sexing trials

In total, sex was assigned to 105 birds in the field, 58 by observer 1 and 47 by observer 2. In comparison with the sex assigned to these individuals by DNA (n = 71) and discriminant function analysis of biometrics (n = 36), 82% of birds were correctly sexed (Fig. 2, Table 5).

Table 3. Results of a logistic regression model used to identify the plumage traits (see Table 1 for details) that vary between male and female Black-tailed Godwits. Sex was assigned using molecular techniques.

Variable	β	Wald	*P*
Grey in back	0.06	4.99	0.026
Breast redness	1.86	7.61	0.006
Head colour	1.98	6.48	0.011

Overall model fit: $\chi^2_3 = 50.9$, $P < 0.001$.

Observer 1 sexed both sexes equally accurately; observer 2 was slightly better at sexing males than females, but the proportional difference between observers was not significant (G-test: G = 0.029, $P > 0.05$).

Table 4. Sex differences in the three plumage traits used to separate male and female Black-tailed Godwits. Chi-squared tests were used to test differences in proportions of scores and Mann–Whitney tests for percentages. See text and Table 1 for details of scoring.

		Males	Females	χ^2	*U*
Headstripes	Black	35	13	25.9**	
	Grey	3	26		
Breast redness	Dark	17	3	26.9**	
	Medium	21	19		
	Pale	0	17		
Grey in Back	% (se)	6.6 (1.8)	25 (3.3)		269.5**

**$P < 0.001$.

Table 5. Accuracy of sexing Icelandic Black-tailed Godwits in the field.

	Birds correctly sexed [a] (% (*n*))	
	Observer 1	Observer 2
Male	83 (35)	90 (30)
Female	87 (23)	59 (17)
Overall	84 (58)	79 (47)

[a]The percentage of individually marked birds of known sex that were correctly sexed by two separate observers.

DISCUSSION

Being able to assign sex accurately is necessary for studies comparing ecological and behavioural sex differences, but many species show only limited sexual dimorphism in size or plumage traits. Here we compared behavioural, molecular, biometric and field-based methods for sexing Black-tailed Godwits in order to develop sexing tools for the species and to investigate the feasibility of such validation processes for other species. Our results suggest that molecular sexing of a small number of individuals, in conjunction with assessments of sex-based variation in biometric or plumage traits, can provide a means of rapidly improving the accuracy of current sexing techniques. These molecular techniques are increasingly inexpensive and widely used, and only a small number of feathers is required.

The success rate in extracting DNA from feathers for molecular analysis was high (only 10 of the 152 samples tested gave no bands in the PCR, despite feathers having been stored at room temperature for, in some cases, over two years). The accuracy of this molecular method is also likely to be high since two sets of primers were used and were found to give entirely consistent (and repeatable) results. If we assume, as the analysis of birds seen copulating suggests, that the DNA analysis sexed all birds correctly, then 98% (82 of 84) individuals were correctly assigned as male or female using the biometric data. The two individuals that were incorrectly assigned were shown to be genetically female by molecular analysis, but lie well within the size range of most males, in terms of both bill and wing length. 'Sex reversal', where ZW (i.e. genetically female) individuals develop physically as males (i.e. showing the ZZ phenotype), is a documented phenomenon in poultry (Lewis & Long 1992, Jacob & Mather 2004) and cannot be completely excluded as an explanation for the two incorrectly assigned female godwits. However, it seems more likely that their measurements simply reflect the naturally overlapping distributions of male and female size ranges (Fig. 1).

When the discriminant model was run on the larger sample (n = 808) the rate at which sex could be assigned on the basis of biometric measurements with 95% certainty was 74%. As this is a very big sample of birds, caught at different times of the year in three different countries, it is unlikely that the variation in the population is much higher. In particular, many of the birds caught were on spring passage in Iceland or autumn passage in England, during which birds from throughout the population can occur in the same flocks (Gunnarsson *et al.* 2005). Thus any geographic variation in the population will have been incorporated in these analyses. Thus 74% is likely to approach the true proportion of Black-tailed Godwits that can be reliably sexed using biometrics. Inaccuracies in recording biometric information, including variation amongst individual measurers (Gosler *et al.* 1995), are likely to be the major constraint on sexing birds using this method.

When sexing birds in the hand using plumage characteristics, 86% (of 77 DNA-sexed birds) were correctly sexed. Again, a slightly higher proportion of males was sexed correctly (90% against 82% for females). For the field trials, the overall success rate was 82%, with 87% success for males but only 73% for females (Fig. 2). This suggests that visual estimation of relative size in the field, complemented by breeding plumage characteristics, can be used to sex godwits as accurately as biometrics recorded in the hand. In winter plumage, however, variation in size in the absence of breeding plumage is likely to be insufficient for sexing in the field.

The values given here refer specifically to *islandica* Black-tailed Godwits. The overall comparison suggests, however, that for the many species with minor sexual dimorphism, sexing tools can readily be developed from an initial sample of individuals sexed by accurate molecular methods. Such methods do not require blood samples but could rely on the far less invasive technique of sampling feathers. In general, only one or two body feathers (with the feather base attached) will be required for molecular analyses. For many species, current sexing methods rely on biometric variation in a relatively small sample of birds (often from a restricted part of the species range) sexed through dissection, and variation in plumage characteristics is rarely described (Prater *et al.* 1977). The increasing availability of DNA sexing tools means that a relatively small number of samples, combined with the types of analytical approaches outlined here, can improve our ability to accurately sex many more bird species on the basis of morphological variation in the future.

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