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Phenotypic Sexing of Marbled Godwits (*Limosa fedoa*): A Molecular Validation

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Abstract.—Marbled Godwits (*Limosa fedoa*) do not present obvious sexual dimorphism, although females are known to be slightly larger than males, on average. During the nonbreeding seasons between August 2007 and April 2009, a noninvasive technique was tested that might permit phenotypic identification of sex in a large sample of individuals from the most important wintering area, Guerrero Negro, México. Blood samples ($n = 577$) were used for molecular sexing. For phenotypic sexing, culmen length was selected because it had the greatest sexual dimorphism. A normal distribution curve for culmen length of each sex and the overlapping area between the two curves was calculated and used to minimize the probability of error in identifying the sex of each individual. Using the criterion that males had a culmen length ≤ 106 mm and females ≥ 108 mm, the sex of 96.4% of the individuals would be correctly identified, 2.8% would be regarded as indeterminate and 0.8% would be sexed erroneously. A discriminant analysis using bill length and other commonly used phenotypic measurements did not improve success in predicting sex. The results indicate that culmen length is a useful measure for sex determination in Marbled Godwit. Identification of the sex of Marbled Godwits in the field offers new possibilities to study life histories of both sexes. In Guerrero Negro, the sex ratio of Marbled Godwits was heavily male-biased (2:1), suggesting latitudinal segregation by sex. Received 14 March 2013, accepted 1 May 2013.

Key words.—culmen length, *Limosa fedoa*, Marbled Godwit, molecular sexing, phenotypic sexing, sex ratio.

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For shorebirds, the difficulty in identifying the sex of the individuals is considered to be an important reason why some aspects of sex differences in ecological processes are poorly understood (Durell 2000). Some studies have tried to overcome this difficulty by using measurements of parts of the body that differ between males and females (Wood 1987). For example, different studies have demonstrated that for some small species in the genus *Calidris* (e.g., Western Sandpiper [*C. mauri*; Page and Fearis 1971], Dunlin [*C. alpina*; Page 1974], Least Sandpiper [*C. minutilla*; Butler and Kaiser 1995] and Purple Sandpiper [*C. maritima*; Hallgrímsson *et al.* 2008]), the length of the culmen provides a reliable way to differentiate the sex of the birds, as females are generally larger in monogamous sandpipers. Recently, various studies have addressed the

difficulty in identifying the sexes by complementing the use of morphologic measurements obtained in the field with molecular techniques related to the sex chromosomes (Griffiths *et al.* 1998; Baker *et al.* 1999; Gunnarsson *et al.* 2006; Ottvall and Gunnarsson 2007). This approach has made it possible to test sexing protocols that are practical, efficient and minimally intrusive or noninvasive (Gunnarsson *et al.* 2006). Thus, having a reliable field technique to determine the sex of the individual expands the possibilities for performing comparative studies that address sexual differences in behavior, ecology and evolution (Ellegren and Sheldon 1997; Baker *et al.* 1999; Gunnarsson *et al.* 2006), and is of great value (Hallgrímsson *et al.* 2008).

In the Marbled Godwit (*Limosa fedoa*) females are larger than males (Gratto-Trevor 2000), but otherwise are very similar. To

identify the sex of individuals of this species, a combination of color of alternate plumage and size of the exposed culmen has been suggested (Gratto-Trevor 2000). However, this technique is limited to the breeding season, because in basic plumage in the non-breeding season, the sexes are very similar, and the probability of assigning sex using bill length dimorphism alone has not been specified. Therefore, our objective was to develop a noninvasive and cost effective technique that permits the correct identification of the sex of Marbled Godwits in the field at any time of the year using only phenotypic measurements. As this requires the sex of the birds to be known beforehand to validate morphological sexing, we determined gender in a large sample of Marbled Godwits using molecular sexing.

METHODS

Sample Collection

Marbled Godwits ($n = 770$) were caught by cannon-netting between August 2007 and April 2009 in Guerrero Negro, Baja California Sur, México. This site is the most important wintering site in the world for Marbled Godwits, wintering nearly 50% of the global population (Gratto-Trevor 2000; Melcher *et al.* 2006). The following phenotypic data were obtained from these specimens using electronic calipers (± 0.1 mm): 1) exposed culmen length; 2) head (total head length and bill minus exposed culmen length); and 3) tarsus length. Body mass was also measured using an analytical balance (± 0.1 g). Blood samples of two to three drops from the brachial wing vein were collected with a blood lancet and stored on Whatman filter paper in an Eppendorf tube at -10°C for molecular analysis.

Molecular Sexing

We captured and collected blood samples from 770 individuals, for molecular analyses for sex determination. However, we only have positive and reliable results for 577 individuals. From this last sample ($n = 577$), we randomly selected 300 individuals to develop the discriminant function and phenotypic analysis with exposed culmen length. With the remaining sample ($n = 277$), we conducted the technical validation of both the discriminant function analysis (DFA) and the ranges for sex identification established with the exposed culmen length.

Blood samples ($n = 577$) were used to sex Marbled Godwits. These samples were analyzed by the Royal Ontario Museum's Department of Natural History using a molecular method based on the amplification of an in-

tron in the conservative CHD1 gene located on the sex chromosomes (the length of this intron differs between the copies on the Z and the W chromosomes; Griffiths *et al.* 1998). The DNA template was obtained from the blood samples using an alkaline extraction (Rudbeck and Dissing 1998). A portion of the filter paper with the blood sample was submerged in 20 μl of 0.2 M NaOH in a 1.5 ml tube and then incubated at 75°C with agitation for 190 min. The solution was then neutralized by the addition of 180 μl of a 0.04 M Tris-HCl pH 7.5 solution. The primers used for sexing the birds by polymerase chain reaction (PCR) were adapted from Fridolfsson and Ellegren (1999) and modified for shorebirds (O. Haddrath, pers. commun.). The multiplex PCR reactions were each carried out in a total volume of 12.5 μl containing 1 μl of DNA template, 0.5 μmol of each of the primers F2602 and R2669, 0.05 μl or 0.25 units of Taq DNA polymerase, 0.28 μl of a 10 mM dNTP solution, 1.25 μl of buffer (10X buffer consisted of 100 mM Tris-HCl pH 8.3, 0.5M KCl and 25 mM MgCl_2) and 8.92 μl of water. The PCR profile began with an initial denaturing temperature of 95°C for 5 min, followed by a 10-cycle touchdown protocol with an initial cycle of 94°C for 30 sec followed by 60°C for 30 sec and then 72°C for 45 sec, with the annealing temperature of each subsequent cycle decreasing by 1°C . The conditions of the final touchdown cycle were repeated for an additional 40 cycles followed by a final DNA extension phase of 5 min at 72°C . The amplified products were separated by electrophoresis in 1% agarose gels stained with ethidium bromide, and the results were visualized using a UV light table.

Phenotypic Analysis

Since 75% ($n = 577$) of the samples that were sexed molecularly had a positive result, 300 were randomly selected. A DFA was carried out on bill, head and tarsus length to provide a model for sexing Marbled Godwits using biometrics. These measurements do not fluctuate as much as mass, which was not included in this analysis. Sex was assigned by DFA to those birds with probabilities of 95% or more of belonging to a particular sex.

To select the most sexually dimorphic variable for possible phenotypic sexing. We conducted Student's t-tests of differences in culmen length, head length and tarsus length between sexes in the 300 individuals selected randomly. Normality tests (Kolmogorov-Smirnov) were applied to the six groups of data (both sexes and three measures) to justify the use of the normal distribution (Zar 1999).

Because culmen length was found to be the most sexually dimorphic character, a normal distribution curve for each sex was calculated and the overlapping area between the two curves was determined using the sample of 300 birds. To minimize the probability of error, the range of sexually indeterminate individuals, or birds in which sex differentiation was unequivocal (e.g., small females or big males), was determined. The objective was to maximize the probability of success in identifying the sex of each individual (Page and Fearis 1971).

With the individuals of known sex ($n = 277$), that were not included in the development of the models, the effectiveness of phenotypic sex identification was assessed (Fowler *et al.* 1998). In these cases, sex was determined based on the length of the culmen and with the DFA, and these results were then compared with the real sex (via molecular analysis). Thus, the success and failure rates of both phenotypic techniques and the percentage of individuals that were correctly sexed were determined (Fowler *et al.* 1998).

RESULTS

Molecular Sexing

Of the 300 individuals that were sexed molecularly, 194 were male (65%) and 106 were female (35%). Primers produced entirely consistent results, and each PCR was performed at least twice and found to be 100% repeatable. Overall, exposed culmen length, head length, and tarsus length were significantly different between the sexes (Table 1). Individuals identified molecularly as females were on average larger than males for all morphologic features measured.

Sexual dimorphism was most evident in culmen length, which was on average 21.9% larger in females than males. Head length had the smallest dimorphism as females were just 3.9% larger than males on average (Table 1). Therefore, we only considered the length of the culmen in the development of the phenotypic technique for sex identification. For each sex, the culmen distribution was normal (K-S $d = 0.064$, $P \gg 0.2$; K-S $d = 0.083$, $P > 0.2$; for males and females, respectively).

Phenotypic Sex Determination

The DFA incorporating bill and head lengths correctly classified 96% of females

($n = 102$) and 96% of males ($n = 187$). In total 96% of all individuals ($n = 289$) were correctly classified to sex. The addition of tarsus length did not improve the probability of correct sex identification and was therefore excluded from the analysis. The estimated discriminant functions were: for females, $D_F = 4.63 \times \text{bill length} + 13.86 \times \text{head length} - 557.15$; and for males, $D_M = 3.78 \times \text{bill length} + 13.37 \times \text{head length} - 446.56$. The effectiveness of the discriminant function was tested with the sample of 277 birds; the DFA classified the sex of 96% of 277 Marbled Godwits at the 95% level of assignment.

The comparative approach using the normal curve generated with the mean and the standard deviation of culmen length for each sex. This allowed us to determine that the phenotypic range of the individuals that maximized the correct identification of sex and minimized the percentage of sexually indeterminate individuals in the overlap zone was between 106 and 108 mm in exposed culmen length. In total, 97.5% of males and only 1.6% of females had a culmen length ≤ 106 mm; 96.4% of the females and only 0.8% of males have a culmen length ≥ 108 mm, and finally 1.7% of males and 2.0% of females had culmen lengths between 106.1 and 107.9 mm and were considered to be of indeterminate sex (Table 2; Fig. 1). Using the criteria developed above, on average, sex was identified correctly in 97% of the birds, 1.8% were indeterminate and 1.2% were classified erroneously to sex (Table 2). To further validate this phenotypic method, we applied it to the 277 birds sexed previously. The sex of 267 individuals in this sample was determined correctly (96.4% success), three birds were considered of indeterminate sex (one female and two males) and seven birds were sexed erroneously.

Table 1. Mean and standard deviation of the phenotypic comparisons between molecularly-sexed male and female Marbled Godwits ($n = 300$). The Students t -values (t), significance values (P) and the percent difference between the sexes (FM) are shown. Measurements are means ($\pm$ SD).

Measurement	Female ($n = 106$)	Male ($n = 194$)	t	P	FM (%)
Culmen length (mm)	118.38 (± 5.76)	97.08 (± 4.56)	32.86	< 0.01	21.94
Head length (mm)	40.87 (± 1.75)	39.33 (± 1.67)	7.51	< 0.01	3.92
Tarsus length (mm)	77.17 (± 4.20)	71.47 (± 3.61)	12.31	< 0.01	7.98

Table 2. Effectiveness of gender determination using Discriminant Function Analysis (DFA) on three phenotypic measurements vs. culmen length of Marbled Godwits. Percentages are indicated in parentheses.

Parameter	Discriminant Function Analysis		Technical Validation (DFA)		Phenotypic Technique with Culmen		Technical Validation (Culmen)	
	Males	Females	Males	Females	Males	Females	Males	Females
Correctly identified	187 (96)	102 (96)	179 (97.2)	87 (93.5)	189 (97.5)	102 (96.4)	179 (97.4)	88 (94.6)
Misidentified	4 (4)	7 (4)	5 (2.8)	6 (6.5)	2 (0.8)	2 (1.6)	3 (1.6)	4 (4.3)
Indeterminate	—	—	—	—	3 (1.7)	2 (2)	2 (1)	1 (1.1)
Total	194	106	184	93	194	106	184	93
Sexes Combined								
Correctly identified	289 (96.3)		266 (96.0)		291 (97)		267 (96.4)	
Misidentified	11 (3.7)		11 (4.0)		4 (1.8)		7 (2.5)	
Undetermined	—		—		5 (1.2)		3 (1.1)	

DISCUSSION

The results confirm the effectiveness of combining a molecular technique for the identification of sex with phenotypic characters that show variation in relation to the sex of the individuals, because both of these provide credible and useful tools for sexual identification in birds with limited dimorphism (Baker *et al.* 1999; Gunnarsson *et al.* 2006; Ottvall and Gunnarsson 2007) like the Marbled Godwit. Because the blood was collected on filter paper lacking a DNA preservative, it was not possible to sex the entire sample of 770 birds for which blood samples were available. However, this can be largely accomplished using just culmen length.

The use of the normal distribution of the measurement of one character (in this case culmen length) to try to establish the range that separates the sexes has not been popular, although it has proven very useful for certain species like the Western Sandpiper (Page and Fearis 1971). Discriminant functions that incorporate several phenotypic variables to allow calculation of a specific discriminant score for each bird have been used extensively in various studies to sex weakly dimorphic species with good results (Brennan *et al.* 1991; Gunnarsson *et al.* 2006; Ottvall and Gunnarsson 2007).

In the present study, the results of both phenotypic methods (DFA and culmen length range) to correctly assign sex of individuals of Marbled Godwits showed the same accuracy. Use of the more computationally intensive DFA with two or three sexually dimorphic phenotypic characters did not improve the percentage of individuals whose sex was correctly assigned (Table 2). In this case, the use of the normal frequencies of culmen length provides a good method to predict the sex of individuals for this shorebird, especially given the simplicity of the method and the demarcation of a small overlap zone in culmen length where the sex of birds cannot be distinguished. Therefore, it seems to be a good discriminating factor when sexing birds, as reflected by a bimodal distribution believed to represent a normal distribution curve for each sex (Hallgrimsson *et al.* 2008).

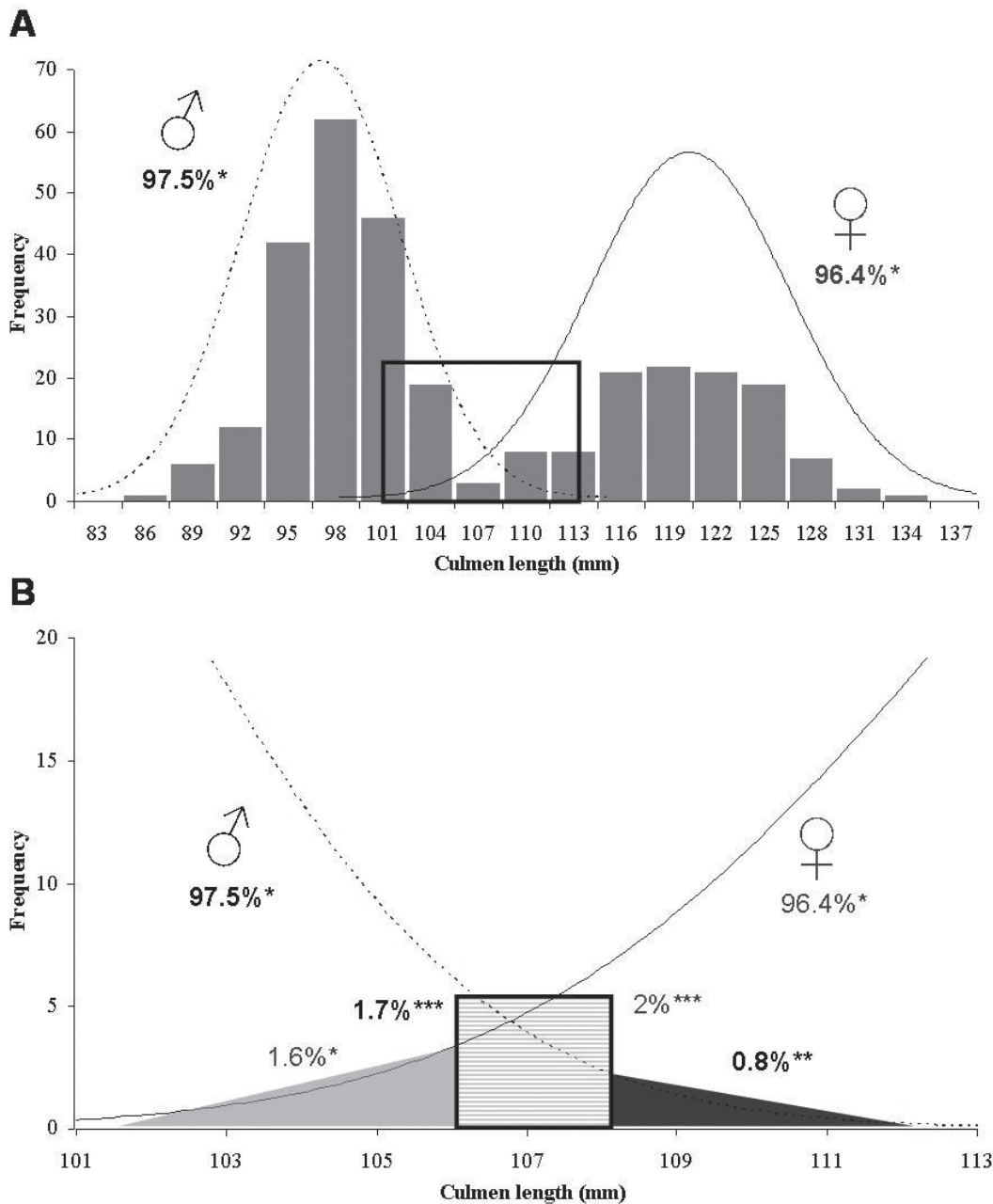


Figure 1. Culmen length distribution of Marbled Godwits ($n = 300$) that were captured and used to establish the criteria for the identification of sex using culmen length. (A) The frequency histogram and the normal distribution curve for each sex. (B) Overlapping area between the two curves, and proposed intervals for the phenotypic identification of sex are indicated: (*) percent effectiveness of sex identification, (**) percent misidentified, and (***) percent undetermined individuals by sex.

It is important to emphasize that the phenotypic technique developed for the identification of the sex of Marbled Godwits using culmen length came from a large sample size ($n = 577$) of individuals that were captured at different times of the year (August 2007-April 2009; Appendix) and at the most important wintering site of this species. This is the reason

why the ranges of culmen length proposed in this study for the identification of sex are robust.

Because classification techniques like DFA are usually much more effective in separating sexes on the sample on which they were focused, it is necessary to test their efficacy on an independent sample. The same concern applies to phenotypic sexing methods. Both methods had the same accuracy (96%) in correctly identifying sex when applied to an independent sample of known sex. This matches the *a priori* success rate when assigning sex in the large sample of molecularly sexed Marbled Godwits, demonstrating that using culmen length to sex Marbled Godwits is widely applicable. It is important to note that inaccuracies in recording phenotypic information are likely to be the major constraint for sexing birds using this method (Gunnarsson *et al.* 2006), but this can be ameliorated by having only one or very few trained observers take measurements of culmen length.

The ability to correctly identify sex of Marbled Godwit individuals in the field offers new possibilities to study different scenarios related to the life history of the sexes. For example, the proportion of males (2:1 with more males than females) observed in this study indicates a possible latitudinal segregation by sex, differential use of resources, and behavioral differences between sexes, among others (Baker *et al.* 1999; Gunnarsson *et al.* 2006); these are topics for which information is currently unavailable (Gratto-Trevor 2000; Melcher *et al.* 2006). The differences in body size of both sexes are apparently related to the mating system (Sandercock 2001). The smaller size of Marbled Godwit males facilitates territorial displays that include acrobatic flights for the formation of pairs (Gratto-Trevor 2000). Thus, the smaller size of the males (correlated with the size of the culmen) would provide them greater agility, positively influencing their fitness (Figueroa 1999). The larger the size of females, the more fertile they are (Sandercock 1998). Individual marking of large samples of known sex birds in long term studies will also enable estimation of annual survival of

both sexes, and possibly help to explain the ecology of possible sex-specific migration strategies in Marbled Godwits.

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Appendix

Appendix. Information about Marbled Godwits that were captured with cannon-nets on the Guerrero Negro wintering area and molecularly sexed.

Month	Year	Site	Individuals Captured
September	2007	Guerrero Negro Lagoon	20
October	2007	Saltwork (ESSA)	15
January	2008	Guerrero Negro Lagoon	37
February	2008	Guerrero Negro and Ojo de Liebre Lagoon	116
April	2008	Ojo de Liebre Lagoon	22
July	2008	Guerrero Negro Lagoon	7
August	2008	Guerrero Negro Lagoon	28
September	2008	Guerrero Negro Lagoon	6
October	2008	Guerrero Negro Lagoon	70
November	2008	Guerrero Negro Lagoon	45
December	2008	Saltwork (ESSA)	49
January	2009	Ojo de Liebre Lagoon	86
February	2009	Guerrero Negro Lagoon	30
March	2009	Saltwork (ESSA)	34
April	2009	Guerrero Negro Lagoon	12
Total			577