A SIMPLIFIED METHOD OF DETERMINING THE SEX OF *PYGOSCELIS* PENGUINS USING BILL MEASUREMENTS

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Received 1 October 2011, accepted 14 June 2012

SUMMARY

POLITO, M.J., CLUCAS, G.V., HART, T. & TRIVELPIECE, W.Z. 2012. A simplified method of determining the sex of *Pygoscelis* penguins using bill measurements. *Marine Ornithology* 40: 89–94.

We examined sexual dimorphism in bill size in adult Adélie Penguins *Pygoscelis adeliae*, adult Chinstrap Penguins *P. antarctica* and adult and juvenile Gentoo Penguins *P. papua* at King George Island, Antarctica, using a DNA-based molecular sexing technique. Bill length and depth were the most consistent dimorphic character examined, with measurements 5.4%–11.5% larger in males than in females, on average. Within breeding pairs sampled, male Chinstrap and Gentoo Penguins had consistently longer and deeper bills than their mates, although bill measurement overlapped between sexes at the population level. We used bill measurements to calculate species- and age-specific discriminant functions that correctly classified 83.2%–96.7% of the individuals in our study following cross-validation. The discriminant functions derived from this analysis provide a practical method of sex determination for all three *Pygoscelis* penguin species in the South Shetland Islands where they breed sympatrically. Posterior probability analysis can also be used to identify individuals that are likely to be incorrectly classified using discriminant function analysis, allowing DNA-based tests for gender to be reserved for targeted use. Furthermore, we report raw morphometric data to facilitate future analysis and discriminant function improvement.

Key words: Discriminant function analysis, sexing, Adélie Penguin, Chinstrap Penguin, Gentoo Penguin, *Pygoscelis*

INTRODUCTION

While some avian taxa have conspicuous sexual variation in plumage, penguins have monomorphic plumage and are difficult to sex by direct observation. Methods of sexing penguins have focused on dissection, cloacal examination, behavior cues, morphometric analysis and, more recently, molecular techniques (Ainley & Emison 1972, Scolaro *et al.* 1987, Williams 1990, Costantini *et al.* 2008). Penguins exhibit a slight sexual size dimorphism, with males tending to be larger in body, bill and flipper size (Davis & Renner 2003). This has led several researchers to calculate species-specific discriminant functions to classify the sex of penguins based on single or multiple morphological characters (Williams & Croxall 1991, Zavalaga & Paredes 1997, Renner *et al.* 1998, Setiawan *et al.* 2004). While DNA-based molecular techniques are considered to be more reliable than morphometric analysis (Hart *et al.* 2009), discriminant functions can provide a quick, minimally invasive and cost-effect method of sex classification (Dechaume-Moncharmont *et al.* 2011).

Previous studies have calculated discriminant functions for the three *Pygoscelis* penguin species: the Adélie (*P. adeliae*), Chinstrap (*P. antarctica*) and Gentoo Penguin (*P. papua*) (Scolaro *et al.* 1987, Kerry *et al.* 1992, Amat *et al.* 1993, Renner *et al.* 1998). However, none of these studies has validated methods of sex classification using DNA-based molecular sexing. These studies also differ in their methods of statistical validation and in the number and type of morphological characters used to assign sex, both within and across species. In addition, there is evidence that morphometric traits can vary between geographically distinct *Pygoscelis* penguin populations, and thus population-specific discriminant functions are often required (Kerry *et al.* 1992, Renner *et al.* 1998).

The objective of this study was, first, to identify male and female adult Adélie Penguins, adult Chinstrap Penguins and adult and juvenile Gentoo Penguins at Admiralty Bay, King George Island, Antarctica using a DNA-based molecular sexing technique. Second, we aimed to assess the extent of sexual dimorphism in each group and provide discriminant functions based on morphological characters that can be used to identify males and females in future studies. In addition, we wished to provide a method to calculate the classification accuracy of discriminant functions at the individual level. We restricted the morphological character examined in our study to bill measurements as these are the most common measurements reported in the literature; are easily repeatable, with well-defined anatomical landmarks; and also tend to exhibit the greatest degree of sexual dimorphism (Davis & Renner 2003).

STUDY AREA AND METHODS

Captures and measurements

We conducted fieldwork within the Antarctic Specially Protected Area (ASPA) no. 128 along the western shores of Admiralty Bay, King George Island, South Shetland Islands, Antarctica (62°10'S,

58°27'W). All three species of *Pygoscelis* penguins can be found breeding sympatrically at this location (Trivelpiece *et al.* 1987). While this area has been the site of a long-term study of the breeding biology and population dynamics of a population of *Pygoscelis* penguins since the late 1970s, discriminant function-based morphological sexing has never been applied to these populations. During the late incubation and early chick-rearing period (December) of 2010, we captured each member of 11, 15 and 10 actively breeding pairs of adult Adélie, Chinstrap and Gentoo Penguins, respectively. In addition, we supplemented these pairs with nine breeding adult Adélie Penguins, four breeding adult Gentoo Penguins and 18 non-breeding juvenile Gentoo Penguins (approximately 1 year old and identified by white head patches that did not reach the eye, incomplete white eye-rings and the lack of a brood patch) (Trivelpiece *et al.* 1985). Using calipers, we measured bill (culmen) length (BL), bill depth (BD, taken through the center of the nostrils) and bill width (BW, taken across the center of the nostrils) to an accuracy of 0.1 mm. All measurements were conducted by the same person (MJP). In addition, we collected one or two breast feathers and, in some cases, a single tail feather from each individual to facilitate molecular sexing.

Molecular sexing

DNA was extracted from two breast feather calamus per bird using QIAGEN DNEasy Blood & Tissue 96 Well Kits (QIAGEN Ltd., West Sussex, UK). Each calamus was finely sliced using a sterile razor blade. When only one calamus was available, tissue from the inside of a tail feather was also used. The manufacturer's protocol was followed with the following modification: during the incubation step, 30 µL proteinase K was added to each sample with 180 µL buffer ATL and incubated at 56°C for 48 h. The extracted DNA was stored in 400 µL buffer AE at -20°C.

DNA sexing was carried out using a multiplex consisting of three primers: P0, P2 and P8 (Han *et al.* 2009). PCR amplifications were carried out in 8.5 µL reactions containing 4 µL 2X Multiplex PCR Master Mix (QIAGEN), 2.5 µL template DNA and 2 µL of the multiplex (2 µL of each primer at 100 µmol/L made up to 1000 µL with sterile water). The thermal cycling conditions were: 95°C for 5 min; 35 cycles of 95°C for 30 s, 53.5°C for 90 s, 72°C for 30 s, followed by a final extension phase at 72°C for 10 min.

The amplified product was electrophoresed through a 2% agarose gel for 1 h at 125 V. Products were detected using ethidium bromide staining and ultraviolet transillumination. Males, the homogametic sex (ZZ), had a single band on the gel due to a single amplified fragment approximately 400 bp long. This corresponds to a region of the CHD-Z gene that is amplified by the P2 and P8 primer pair. Females, the heterogametic sex (ZW), had two bands on the gel at approximately 400 bp and 500 bp. These correspond to amplified regions of the CHD-Z and CHD-W genes, respectively. The partial CHD-W fragment is amplified by the P0 and P2 primer pair.

Using these molecular techniques, we successfully sexed 97 of the 103 individuals tested. The six individuals that could not be sexed were all breeding adults with eggs or chicks whose mates (four males and two females) had been successfully sexed using DNA. While same-sex mating behaviors have been observed in penguins (Davis *et al.* 1998), truly same-sex breeding pairs, which last long enough to result in successful reproduction, are likely extremely rare (Young *et al.* 2008, Pincemy *et al.* 2010). Therefore, we assumed that the eight individuals that could not be sexed using DNA were the opposite sex of their mates.

Statistical analysis

We compared morphological measurements between males and females using *t*-tests and calculated an index of sexual dimorphism (DI, %) using the mean morphological measurement of males (M) and females (F) as $DI = 100 \times (M - F)/F$ (Greenwood 2003). Next, we conducted separate stepwise discriminant analyses to select the morphological variables (BL, BD, BW) that had significant influence on classification of males and females for each group. We used the *F*-test of Wilks' λ value as a criterion to enter the variable contributing the most, or to remove the variable contributing the least, discriminatory power to the model. The equality of group covariance matrices was tested with Box's *M*-test (Manly 2005). Pearson's correlation matrices found that all values were less than 0.66, indicating there was no multicollinearity between bill characters from our four sample groups (Zar 1984; Arnould *et al*. 2004). Selected variables were used to calculate discriminant functions, and individuals were classified as male or female on the basis of their discriminant score (D). We calculated the percentage of correct classification before and after a cross-validation or "leave-one-out test" (Dechaume-Moncharmont *et al.* 2011).

Similar to Zavalaga *et al.* (2009), we also calculated the posterior probability (PP) of membership of each bird as the probability that an individual with a particular value of D is, or is not, likely to be a male, following Bayes' rule. Values of PP and D were then fitted to a logistic curve to create group-specific functions that can be used to calculate the level of classification accuracy (PP) of an individual for any given D score. Statistical calculations were performed using SAS (version 9.1). All tests were two-tailed, and significance was defined at the $P < 0.05$ level.

RESULTS

Sexual dimorphism

While there was overlap in some morphological measurements, male penguins tended to have larger bills than female penguins in each group examined (Table 1; raw bill measurement data are in Appendix 1 available online). In adults, bill measurement were 8.1%–9.9% larger in Adélie Penguin males, 8.8-11.5% larger Chinstrap Penguin males and 5.4%–10.3% larger in Gentoo Penguin males, relative to females. BL and BD, but not BW, were larger in juvenile males than in female juvenile Gentoo Penguins (Table 1). We also found that, within our sample of breeding pairs of Gentoo and Chinstrap Penguins, males consistently had a larger BL and BD than their female mate. Within-pair comparisons in Adélie Penguins were less diagnostic, with males having the larger BL and BD in 63.6% and 90.9% of all pairs, respectively.

Discriminant function analysis

Stepwise discriminant analysis selected BL and BD as the two variables that best classified adult male and female penguin in all three species (Table 2). There was low overlap between males and females when examining these two bill measurements (Fig. 1). The classification accuracy of the linear discriminant function for adult Adélie Penguins was 90.3% and 83.8% after cross-validation (Wilk's $\lambda = 0.38$, $P < 0.0001$; Box's $M = 2.32$, $P = 0.5082$). Adult Adélie Penguins with $D \geq 0.000060$ were classified as females

when the posterior probability was set at 0.5 (Table 2). Chinstrap Penguin classification accuracy did not change after cross-validation (96.7%; Wilk's $\lambda = 0.25$, $P < 0.0001$; Box's $M = 5.8$, $P = 0.1197$). Adult Gentoo Penguin classification accuracy was 91.7% and 83.2% after cross-validation (Wilk's $\lambda = 0.44$, $P = 0.0002$; Box's $M = 2.25$, $P = 0.5221$). Adult Chinstrap and Gentoo Penguins with $D \geq 0.000053$ and ≥ 0.000231, respectively, were classified as females at a posterior probability of 0.5 (Table 2). Stepwise discriminant analysis selected BL, BD and BW as the three variables that best classified male and female juvenile Gentoo Penguin (Fig. 1; Wilk's $\lambda = 0.32$, $P = 0.0009$; Box's $M = 11.18$, $P = 0.0830$). The classification accuracy of the linear discriminant function for juvenile Gentoo Penguins was

TABLE 1
Bill size measurements and sexual dimorphism in *Pygoscelis* penguins at Admiralty Bay, King George Island, Antarctica

Group, measurement	Mean ± SD (range)		DI (%)[a]	*t*-test
	Male	Female		
Adélie Penguin - adult (n)	16	15		
Bill length (mm)	40.8±2.1 (37.6-43.7)	37.7±2.5 (33.2-40.6)	8.2	$t = 3.66$, $P = 0.0010$
Bill depth (mm)	19.9±0.7 (18.9-21.1)	18.1±0.9 (16.8-19.6)	9.9	$t = 6.59$, $P < 0.0001$
Bill width (mm)	13.4±0.9 (12.0-15.0)	12.4±1.0 (9.7-14.2)	8.1	$t = 2.88$, $P = 0.0074$
Chinstrap Penguin - adult (n)	15	15		
Bill length (mm)	50.4±1.6 (47.5-53.5)	45.2±3.0 (37.3-49.5)	11.5	$t = 5.97$, $P < 0.0001$
Bill depth (mm)	19.8±0.7 (18.6-21.4)	18.2±0.5 (17.4-19.2)	8.8	$t = 7.02$, $P < 0.0001$
Bill width (mm)	15.0±1.2 (13.8-17.6)	13.7±1.3 (12.1-17.9)	9.5	$t = 2.93$, $P = 0.0066$
Gentoo Penguin - adult (n)	11	13		
Bill length (mm)	48.7±2.6 (44.3-52.0)	44.9±2.2 (41.4-49.2)	8.5	$t = 4.01$, $P = 0.0006$
Bill depth (mm)	17.2±0.9 (16.2-18.9)	15.6±0.7 (14.5-16.4)	10.3	$t = 4.70$, $P = 0.0001$
Bill width (mm)	11.8±0.6 (10.7-12.6)	11.2±0.6 (10.4-12.5)	5.4	$t = 2.55$, $P = 0.0184$
Gentoo Penguin - juvenile (n)	6	12		
Bill length (mm)	46.9±1.0 (46.1-48.2)	43.4±1.9 (39.9-46.5)	8.1	$t = 4.2$, $P = 0.0007$
Bill depth (mm)	15.8±0.6 (15.2-16.8)	14.9±0.5 (14.2-15.8)	6.0	$t = 3.67$, $P = 0.0021$
Bill width (mm)	11.4±0.3 (11.1-11.9)	10.9±0.7 (10.0-12.5)	4.6	$t = 1.99$, $P = 0.0642$

[a] DI (dimorphism index) is the difference in percentage as $100 \times (M - F)/F$, where M is the male measurement and F is female measurement.

TABLE 2
Discriminant and posterior probability of assignment functions for *Pygoscelis* penguins at Admiralty Bay, King George Island, Antarctica

Group	Discriminant function[a]	Posterior probability (male)[b]	Classification accuracy (cross-validated)[c]
Adélie Penguin (adult)	$= 64.03041 - 2.85219BD - 0.25089BL$	$= \frac{1}{1 + \exp(1D - 0.000060)}$	90.3% (83.8%)
Chinstrap Penguin (adult)	$= 120.25754 - 4.10985BD - 0.87985BL$	$= \frac{1}{1 + \exp(1D - 0.000053)}$	96.7% (96.7%)
Gentoo Penguin (adult)	$= 53.19063 - 1.89275BD - 0.47576BL$	$= \frac{1}{1 + \exp(1D - 0.000231)}$	91.7% (83.2%)
Gentoo Penguin (juvenile)	$= 129.0415 - 2.86241BD - 1.14292BL - 3.00143BW$	$= \frac{1}{1 + \exp(1D - 0.060900)}$	94.4% (91.7%)

[a] Bill measurements (mm): BD = bill depth, BL = bill length, BW = bill width
[b] D = discriminant score
[c] Percentage of correct classifications before and after (in parentheses) "leave-one-out" cross-validation.

94.4% and 91.7% after cross-validation. Juvenile Gentoo Penguins with D ≥ 0.060900 were classified as females when the posterior probability was set at 0.5 (Table 2).

DISCUSSION

Pygoscelis penguins at Admiralty Bay, King George Island, exhibited sexual size dimorphism, with males tending to be significantly larger in most measurements (Table 1). BL and BD measurements tended to be the most consistently dimorphic characters for all three species. Within breeding pairs, male Chinstrap and Gentoo Penguins had consistently longer and deeper bills than their mates. However, it is important to note that bill measurement overlapped slightly between sexes at the population level. Given this trend, a larger sample of within-pair comparisons may indicate that relative bill size, while a useful sexing tool in Chinstrap and Gentoo

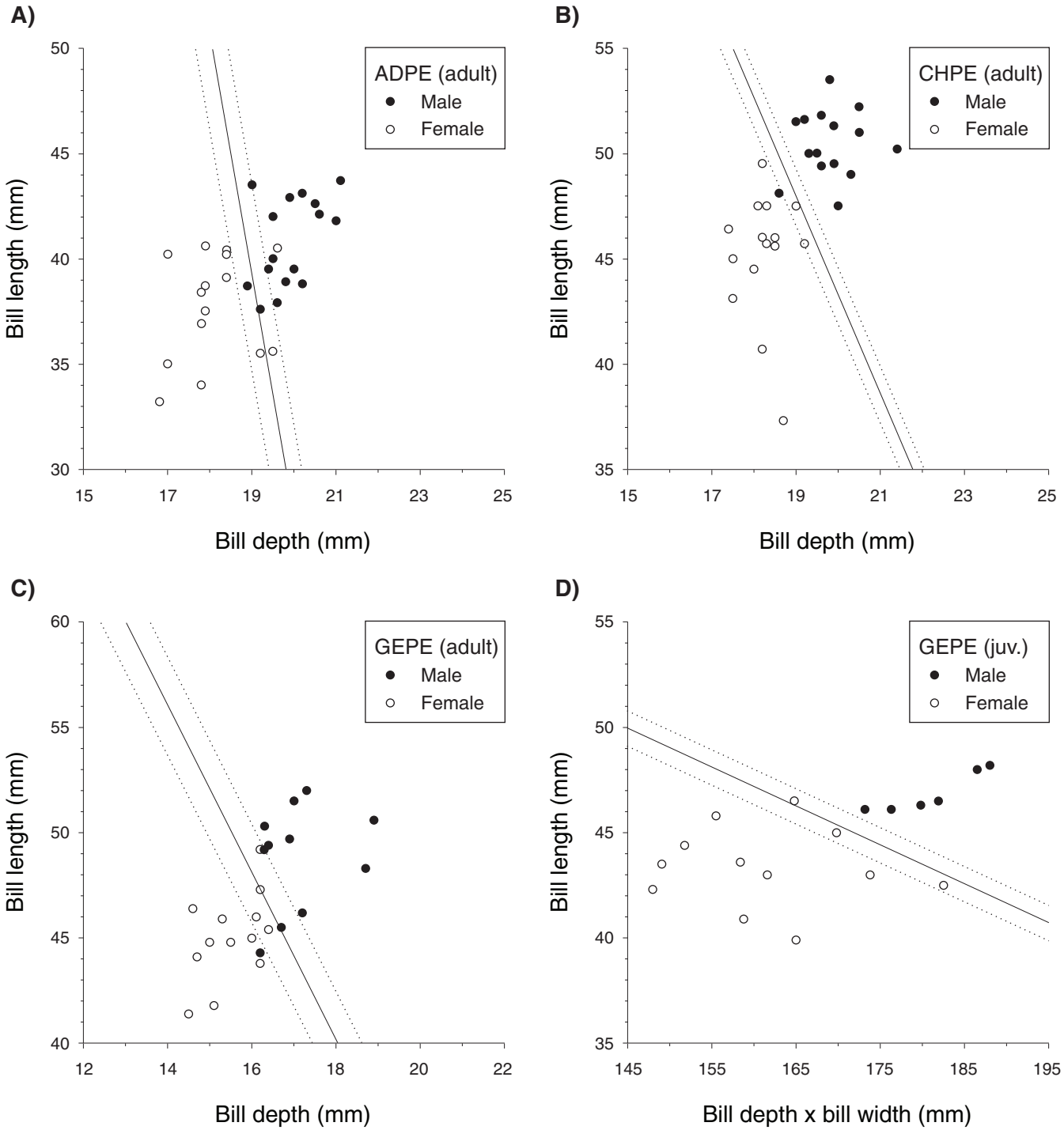

Fig. 1. Bill length and bill depth for breeding adult Adélie (A), Chinstrap (B) and Gentoo (C) penguins, and bill length and bill depth × bill width for juvenile Gentoo Penguins (D) at Admiralty Bay, King George Island, Antarctica. Solid lines represent a 50% posterior probability (PP) of correct sex assignment based on the discriminant functions described in Table 2. All birds above theses lines were classified as males. Dotted lines represent 25% and 75% posterior probabilities.

Penguin pairs, is likely less than 100% reliable. The magnitude of bill-size dimorphism we observed in *Pygoscelis* penguins at Admiralty Bay (5.4%–11.5%) was relatively small in comparison with that of other penguin genera such as *Spheniscus* (7.4%–15.3%) and *Eudyptes* (10.2%–17.8%; Agnew & Kerry 1995). Inter-specific variation in sexual dimorphism may be influenced by environmental factors; body size and BL in penguins tend to decrease with both latitude and environmental temperature (Symonds & Tattersall 2010). The higher degree of sexual dimorphism observed in *Spheniscus* and *Eudyptes* penguins may be driven by the greater importance of foraging-related factors, such as resource limitation and competition, in more moderate-temperature habitats and may thus lead to a greater selective pressure on bill size (Agnew & Kerry 1995). Sexual dimorphism in *Eudyptes* penguins can also be related to male mating displays and mate recognition (Warham 1972).

Observed sexual size dimorphism in avian populations may also be influenced by age structure. Mínguez *et al.* (2001) found that first-time breeding Chinstrap Penguins nesting on the edge of the colony had smaller bills than older, more experienced breeders nesting in central positions. At our study site, one-year-old male Gentoo Penguins have bill measurements similar in size to those of breeding adult females. Therefore, age-specific variation may have the potential to influence measures of sexual size dimorphism if avian morphometric characters continue to grow through early adulthood (Coulson *et al.* 1981, Bortolotti 1984, Mínguez *et al.* 1998).

The discriminant functions derived from this study provide classification accuracies roughly similar to those reported in previous studies of *Pygoscelis* penguins (Scolaro *et al.* 1987, Kerry *et al.* 1992, Amat *et al.* 1993, Renner *et al.* 1998). Unfortunately, due to differences in the number and type of morphological characters used to assign sexes, it is difficult to directly compare our results with these previous studies. Only Amat *et al.* (1993) provides a discriminant function using BL and BD measured in the same manner that is therefore directly comparable to ours. Amat *et al.*'s (1993) discriminant function for Chinstrap Penguins at Deception Island would have correctly assigned sex to 93.3% of the individuals in our data set, whereas the discriminant function derived in this study correctly assigned sex to 96.7% of individuals. This suggests that, at least for Chinstrap Penguins, the discriminant function derived in this study may be applicable to other breeding sites in the South Shetland Islands.

Posterior probability analysis allowed us to determine the relative accuracy of sex assignment for individuals sexed with the discriminant functions derived in this study. This approach can allow researchers to identify individuals with intermediate morphometric characters that are most likely to be incorrectly assigned (Hart *et al.* 2009, Zavalaga *et al.* 2009). Assessing the reliability of individual sex classifications can allow the targeted use of the more expensive and labor-intensive DNA-based molecular tests to definitively assign gender when confidence in the discriminant function is low. For example, Kerry *et al.* (1992), suggests that discriminant functions with an overall success rate of > 80% are acceptable for most purposes. This same cut-off value could be applied to the posterior probability values of individuals, although researchers should be cautious to assign cut-off values appropriate to the degree of sexual dimorphism in their study species (Hart *et al.* 2009). Regardless, DNA testing should be preferentially used in field studies when there is likely to be a small effect size between sexes and sex misclassifications would have a disproportionate overall effect (Hart *et al.* 2009).

While the classification accuracies from this study were similar to previous analyses, the discriminant functions resulting from our study have both advantages and disadvantages. For example, the use of DNA-based methods to validate our methods of sex classification represents a more robust methodological approach than found in previous studies. However, we also used generally smaller group sample sizes to calculate the discriminant functions than previous studies (20–31 individuals vs. 35–55 individuals per group). To help address this issue, our raw measurement data have been included as an appendix to facilitate increased sample sizes and the refinement of discriminant function in the future. An advantage of this study is that we provide a method of estimating the posterior probability of sex assignment for individuals sexed via discriminant analysis, which can allow for targeted use of DNA-based sexing methods. In addition, we used consistent morphological characters across adults of all *Pygoscelis* species in our discriminant functions. Furthermore, BL and BD are the most common measurements reported in avian literature, allowing for a greater application across studies (Davis & Renner 2003, Dechaume-Moncharmont *et al.* 2011). Reducing the number and diversity of measurements required to sex individuals is of practical benefit to researchers, especially in the Antarctic Peninsula region where *Pygoscelis* penguins breed sympatrically. Future work using comparable morphological characters is required to determine how well our discriminant and posterior probability functions perform at other breeding locations.

ACKNOWLEDGEMENTS

Many thanks to the US Antarctic Marine Living Resources program, especially S. Trivelpiece, G. Watters, A. Will, P. Chilton and K. Boysen. Thank you to Raytheon Polar Services, the Laurence M. Gould, the M/V Ushuaia, John Evans and Ethan Norris for transportation and logistical support. Thank you to K. Ciborowski and C. Zavalaga for laboratory and statistical advice. Animal use in this study was conducted under approved animal use protocols from the University of North Carolina Wilmington Animal Care and Use Committee (A0910-20) and in accordance with Antarctic Conservation Act permits provided by the US National Science Foundation (NSF) to S. Emslie (2006-001) and G. Watters (2011-005). Funding was provided in part by a NSF Office of Polar Programs grant to S. Emslie (ANT-0739575). This manuscript was improved through the comments of P. Dann and two anonymous reviewers.

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