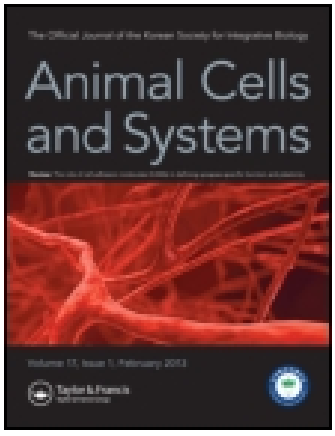

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Sex identification is a fundamental work for studying the behavioural ecology in animals. Although recent molecular sexing techniques have enabled us to distinguish the sexes, it is still convenient to discriminate the sexes with morphological traits especially when surveying colonial animals under harsh field conditions. For chinstrap and gentoo penguins in Antarctica, previous studies developed several morphological discriminant functions, but many studies did not adopt molecular sexing methods for deciding sexes. In this study, we tested previous morphology-based sexing methods to determine their applicability to adult chinstrap and gentoo penguins breeding at Narębski Point (Antarctic Specially Protected Area No. 171) in Barton Peninsula on King George Island. Furthermore, we aimed to develop alternative morphological features to reliably discriminate penguin sexes. Our results showed that the accuracies of previously suggested functions varied among discriminant functions in both species (approximately 64–82%). Here, we developed new functions to discriminate sexes in chinstrap and gentoo penguins, using bill and middle toe size which are easily acquired and less error-prone. The classification accuracy of the discriminant functions derived in this study was >90% for both species. Also, it was successfully applicable to another chinstrap population.

Keywords: morphological sexing; molecular sexing; chinstrap penguin; gentoo penguin

Introduction

Sex identification is essential when studying the behavioural ecology of birds. However, in many penguin species, the sexes are not easily distinguished based on appearance because body colour patterns and body sizes are similar (Agnew & Kerry 1995). Thus, past studies have developed techniques to determine sex via combining several morphological differences in bills or flippers (e.g. Kerry et al. 1992 for the Adélie penguin, *Pygoscelis adeliae*; Hull 1996 for Royal penuigns, *Eudyptes schlegeli*, and Rockhopper penguins, *Eudyptes chrysocome*; Arnould et al. 2004 for the Little penguin, *Eudyptula minor*). Although recent molecular sexing techniques have enabled us to distinguish the sexes, morphological traits must still be used when surveying colonial birds under harsh field conditions.

For chinstrap and gentoo penguins, several morphological features and discriminant functions have been suggested, and many studies have not yet adopted molecular sexing methods (Williams 1990; Amat et al. 1993; Renner et al. 1998; but see Polito et al. 2012 and Valenzuela-Guerra et al. 2013). However, even within the same species, different morphological features are applied for distinct populations, making a universal method difficult (Valenzuela-Guerra et al. 2013). Thus, using molecular sexing, we tested morphology-based sexing methods to determine their applicability to adult chinstrap and gentoo penguins breeding at Narębski Point (Antarctic Specially Protected Area No. 171) in Barton Peninsula on King George Island. Furthermore, we aimed to develop alternative morphological features to reliably discriminate penguin sexes, such as bill and middle toe size, which are easily acquired and less error-prone.

Materials and methods

During the 2013/2014 breeding season, we measured morphological traits with digital callipers and a ruler (0.1 mm accuracy), and sampled blood from 46 and 44 randomly captured adult chinstrap and gentoo penguins, respectively. By observing their nesting and feeding behaviour, we confirmed that the captured individuals were all breeding adults. Among phenotypic parameters, we excluded "maximum gap of the bill" (Renner et al. 1998) and "total length from bill to tail" (Valenzuela-Guerra et al. 2013) since these were difficult to estimate due to the animals' movements and varied largely among repeated measurements. Instead, we used middle toe length, which was used in Adélie penguin sex determination, easily distinguished on the ventral surface from the tarso-metatarsal joint to the end of middle toe claw (Kerry et al. 1992, for measurement; see Figure 1). For analysis, we used bill length, bill depth, flipper length and middle toe length (for measurement pictures of bill and flipper size, see Amat et al. 1993 for chinstrap and Valenzuela-Guerra et al. 2013 for gentoo). To avoid user bias, a single person measured all parameters.

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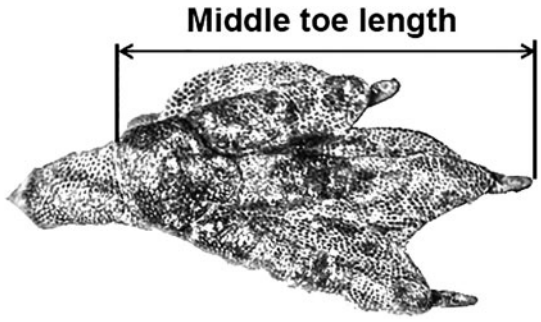

Figure 1. A picture of middle toe length measurement from the tarso-metatarsal joint to the end of the middle toe claw, on the ventral side.

From each individual, we collected blood in a vein in the middle toe and stored in a heparinized tube. Then, we extracted DNA from blood samples using the DNeasy blood and tissue kit (Qiagen) and determined sex using polymerase chain reaction bands with 2550F/2718R primers (see Fridolfsson & Ellegren 1999). Then, we used our molecular sexing results to determine the classification accuracy of morphological sex discriminating methods.

We compared our four measurements (bill length, bill depth, flipper length and middle toe length) between sexes using *t*-tests. We applied our data to previous discriminant functions for chinstrap and gentoo penguins using bill length, bill depth and flipper length (Amat et al. 1993 and Polito et al. 2012 for chinstrap; Williams 1990 and Polito et al. 2012 for gentoo) to test whether these are applicable to our data-set. For each species, we performed stepwise

Table 1. Four size measurements (mm, mean ± SD) and *t*-test results for each parameter in 26 male and 20 female chinstrap penguins, and 22 male and 22 female gentoo penguins.

Species	Sex	Bill length	Bill depth	Flipper length	Middle toe length
Chinstrap (n = 46)	Male	50.6 ± 2.1	19.6 ± 1.2	196.9 ± 6.9	89.9 ± 3.8
	Female	45.3 ± 2.2	17.5 ± 0.8	192.9 ± 6.8	87.3 ± 3.0
		t = 9.83, P < 0.001	t = 7.33, P < 0.001	t = 3.98, P < 0.001	t = 3.61, P < 0.001
		DI = 11.70	DI = 12.00	DI = 2.08	DI = 2.98
Gentoo (n = 44)	Male	47.7 ± 2.4	16.7 ± 1.0	217.3 ± 7.5	92.6 ± 2.7
	Female	43.8 ± 2.0	15.7 ± 1.1	212.4 ± 5.4	86.6 ± 2.7
		t = 5.92, P < 0.001	t = 3.43, P = 0.001	t = 2.49, P = 0.017	t = 7.41, P < 0.001
		DI = 8.90	DI = 6.37	DI = 2.31	DI = 6.93

DI, dimorphism index was calculated as the percentage of the difference between male and female measurement to the female measurement [DI = 100 × (male value – female value)/(female measurement)].

Table 2. Evaluations of previously suggested discriminant functions when applied to our data-sets.

Species	Reference	Study site	Discriminant function[a] (score for determining male)	Accuracy (%)
Chinstrap	Amat et al. (1993)	Deception Island	= 0.213BL + 4.360BD + 0.137FL (>120.85)	78.3
			= 0.409BL + 4.113BD (>99.77)	78.3
	Polito et al. (2012)	King George Island	= 120.25754 – 4.10985 BD – 0.87985BL (<0.000053)	81.7
Gentoo	Williams (1990)	Bird Island (South Georgia)	= 0.922BL + 3.885BD (>112.608)	63.6
	Polito et al. (2012)	King George Island	= 53.19063 – 1.89275BD – 0.47576BL (<0.000231)	75.0

BL, bill length; BD, bill depth; FL, flipper length.
[a]If the discriminant score value is corresponding to the range in parentheses, the individual is male.

Table 3. New sex determination functions by discriminant analyses and classification accuracies from our study on King George Island, Antarctica.

Species	Discriminant function[a] (score for determining male)	Accuracy (cross-validated)[b]
Chinstrap	= 0.369BL + 0.456BD – 26.379 (>–0.008)	90.1% (90.1%)
Gentoo	= 0.228BL + 0.274MTL – 34.899 (>0)	93.2% (90.9%)

BL, bill length; BD, bill depth; MTL, middle toe length.
[a]If the discriminant score is higher than the value in parentheses, the individual is male.
[b]Accuracy is the percentage of correct predictions of our penguin sexes compared to molecular sexing results (cross-validated values indicate the percentage of correct predictions after cross-validation).

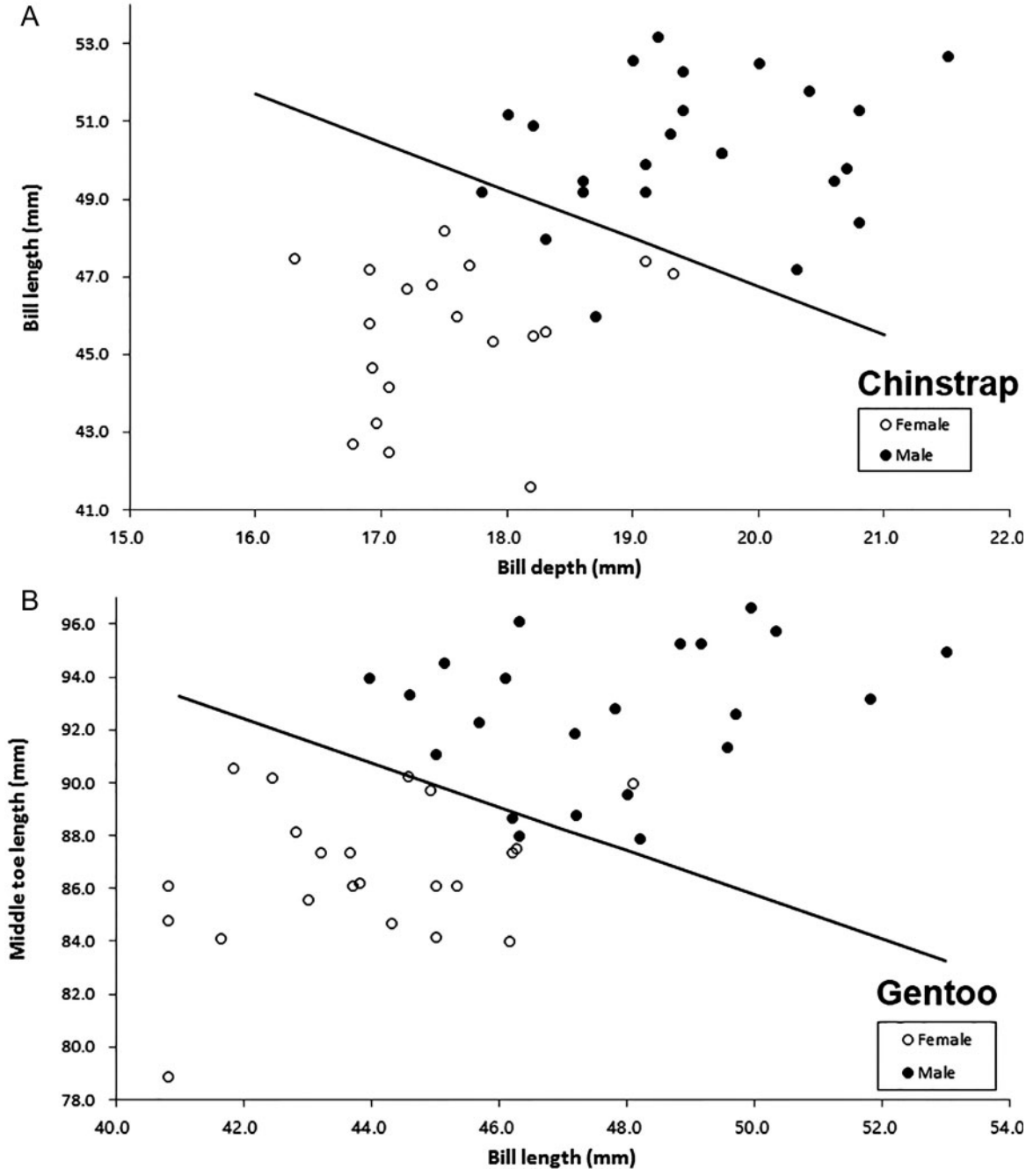

Figure 2. Bill depth and bill length in 46 adult chinstrap penguins (A), and bill length and middle toe length in 44 adult gentoo penguins (B). Note: Filled circles are males and empty circles are females. Solid lines indicate the discrimination function values for determining the sexes.

discriminant analysis (for details, see Polito et al. 2012). We calculated the percentages of classification accuracy of the discriminant functions after leave-one-out cross-validation (Dechaume-Moncharmont et al. 2011) in SPSS (version 18.0). The raw data on penguin measurements can be delivered upon request.

Results and discussion

By molecular sexing, we successfully determined the sex of all individuals: 26 males and 20 females for chinstrap, and 22 males and 22 females for gentoo were identified. In both species, males had higher values than females for all four parameters (Table 1).

When we compared our molecular results with previously suggested discriminant functions, the classification accuracies were 78.3–81.7% for chinstrap and 63.6–75.0% for gentoo penguins (Table 2). Considering that the previous functions were originated from other populations, these results suggest that these functions with bill and flipper sizes are quite convincing. Since the study

population of Polito et al. (2012) was also located on King George Island, high morphological similarities were expected. Among the discriminant functions, the accuracies of Polito et al.'s were the highest and relatively acceptable when applied to our data-set in both species (81.7% for chinstrap and 75.0% for gentoo). The relatively low accuracy (63.6%) of Williams (1990) may be due to the subspecies difference of sizes between northern and southern gentoo penguins.

The new discriminant functions that we developed in this study correctly classified the sex of >90% of individuals in our study populations, and the classification accuracy of the discriminant functions derived in this study was >90% for both species (Table 3). In our results, bill length and bill depth were the most distinguishable variables for chinstrap penguins (Table 3, Figure 2A), in agreement with previous studies. On the other hand, bill length and middle toe length were the most important for discriminating gentoo individuals (Table 3, Figure 2B).

When we applied our discriminant functions to the raw data-set of Polito et al. (2012) obtained on chinstrap pairs, 93.3% (28/30) of individuals were correctly classified. Although this does not indicate that our functions are broadly applicable, we expect that our new discriminate functions are successfully used in this study area with a high accuracy. Also, our study provides that middle toe length measurement may be one of factors in morphological sex determination in gentoo penguins. Future studies should evaluate our discriminant functions using additional chinstrap and gentoo populations.

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