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Field sexing techniques for Fiordland crested penguins (tawaki; *Eudyptes pachyrhynchus*)

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Abstract: Fiordland crested penguins (tawaki; *Eudyptes pachyrhynchus)* lack sexually dimorphic plumage so behavioural cues or bill size have traditionally been used to determine sex in the field. We aimed to identify morphological characters that can be quickly and reliably be measured in the field to accurately sex adult tawaki, and validated these with genetics. We measured five morphological parameters in tawaki (n = 32) from three colonies (Jackson Head, Milford Sound/Piopiotahi, and Codfish Island/Whenua Hou) on the New Zealand South Island. We confirmed sex with a PCR-based molecular assay. Male tawaki are larger in all parameters measured and recursive partitioning trees correctly classify 94% of penguins sampled. In line with Warham (1974) and Murie *et al.* (1991), we propose using bill length (males > 44.5 mm) and bill depth (males > 25.5 mm) but in combination with foot length (males > 113.5 mm) to determine tawaki sex in the field. These morphological parameters are independent of body condition and are easily obtained in the field.

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INTRODUCTION

In many penguin species, males and females occupy predictable roles during the breeding season that dictate their behaviour both on land and at sea (Warham 1974; Williams & Croxall 1991). These differences in behaviour influence energy expenditure, access to resources, and risk of predation (González-Solís *et al.* 2000; Donald 2007; Morrison *et al.* 2017). However, understanding sex-based variations in ecology, foraging behaviour, and demography is confounded by the lack of clear sexually dimorphic traits. *Eudyptes* penguins exhibit strict partitioning of incubation and chick rearing duties that has been used to determine sex during the breeding season, however such traits do not extend into other periods of the annual cycle (Warham 1974; Kriesell *et al.* 2018; Mattern & Wilson 2018).

Fiordland crested penguins (*Eudyptes pachyrhynchus;* hereafter referred to as tawaki*)* lack any obvious external sexual dimorphism (Warham 1974). Previous research by Warham (1974) and Murie *et al.* (1991) suggested behavioural cues, such as nest attendance patterns, along with bill size as reliable metrics for determining sex.

Multiple parameters have been used to sex penguins in the field. Some, such as vent measurements (Boersma & Davies 1987) and cloacal examinations (Samour *et al.* 1983) require expertise. Common morphometric parameters assessed in multiple penguin species include total mass, bill length and depth, head length, wing length, total foot length, and tarsus length. These metrics are used in southern rockhopper penguins (*Eudyptes chrysochome;* Poisbleau *et al.* 2011), northern rockhopper penguins *(Eudyptes moseleyi;* Steinfurth *et al.* 2019), little penguins/kororā (*Eudyptula minor;* Overeem *et al.* 2006), yellow-eyed penguins/hoiho (*Megadyptes antipodes;* Setiawan *et al.* 2004), and African penguins (*Spheniscus demersus*; Campbell *et al.* 2016) but have not been verified for tawaki alongside molecular sexing protocols.

Here we use morphometric and PCR-based molecular sexing protocols to identify morphological characters that are both consistently distinguishable between sexes and can be obtained quickly and reliably in the field. Although Warham (1974) and Murie *et al.* (1991) identified the overall bill shape and size (bill index) to be the most distinguishable metric, this technique was confirmed using behavioural cues only. Confirming a method that is independent of seasonality, behaviour, or body condition would allow accurate sexing of adult tawaki throughout the annual cycle.

METHODS

Study Sites

We sampled tawaki at three sites in southern New Zealand: in south Westland at Jackson Head (43.963°S, 168.611°E); the Harrison Cove colony (44.624°S, 167.913°E) in Milford Sound/ Piopiotahi in Fiordland National Park and the Piopiotahi Marine Reserve; and the Whenua Hou colony (46.760°S, 167.641°E) on the north-eastern coast of Codfish Island/Whenua Hou.

Capture and Sampling Protocols

We captured adult tawaki during 19 September to 5 October 2018. In this period, males remain at the nest while females forage during the day (Warham 1974). We captured assumed males at their nests and intercepted putative females on the beach as they returned at dusk. In total, we sampled 34 adult tawaki: 8 from Harrison Cove (4 male & 4 female), 20 from Jackson Head (9 male & 11 female), and 6 from Whenua Hou (1 male & 5 female).

We recorded total mass (kg), foot length (mm), head length (mm), bill length (mm), and bill depth (mm) (Warham 1972; Murie *et al.* 1991; Figure 1).

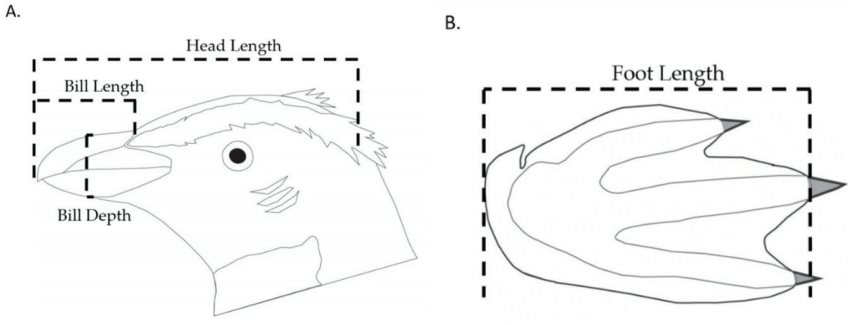

Figure 1. Measurement locations on tawaki (Fiordland crested penguin *Eudyptes pachyrhynchus*) head (A) and foot (B). Head and foot length were obtained using an osteometric board while bill length and depth were taken via digital callipers.

We weighed tawaki using a Pesola 5 kg spring balance to the nearest 10 g. We measured total foot length from the heel to the distal tip of the last pad of the central toe and total head length from the post occipital crest to the tip of the culmen using an osteometric board to the nearest 1 mm (Setiawan *et al.* 2004). Finally, we measured bill length and bill depth using digital callipers (Jobmate J701-2702) to the nearest 1 mm. Following Warham (1972), bill length included the exposed portion of the culmen while bill depth was measured perpendicular to the point of the inter-ramal feather patch (Figure 1).

Molecular Sexing

We collected whole blood (0.1 – 0.5 mL) from the brachial vein using new 25-gauge needles and 1.0 mL tuberculin syringes. Samples were stored in 70% ethanol until field work was completed and extraction procedures began. Total genomic DNA was extracted from each blood sample using standard phenol-chloroform protocols followed by a polymerase chain reaction (PCR). We selected primers (SEX1/SEX2) designed to match conserved exon flanking regions of an intron in the chromo-helicase-DNA binding protein (CHD) gene on the Z (CHD-Z) and W (CHD-W) sex chromosomes in birds (Wang & Zhang, 2009). We chose these based on previously successful sexing of northern rockhopper penguins (Steinfurth *et al.* 2019) as well as southern rockhopper, macaroni, and little penguins (JW *unpubl. data*). Following PCR, we separated amplicons by size by loading the entire 20 µL of each reaction on a 3 % agarose TBE gel stained with ethidium bromide and visualized the bands (BioRad Molecular Imager®) following 200 volt-hours of electrophoresis. Individuals producing a single band were classified as males (ZZ) and those with two bands as females (ZW).

Data Analysis

We analysed data in R (RStudio Team 2006–2018, Version 1.1.442) and employed the Lilliefors (Kolmgorov-Smirnov) test to assess all variables for normality and used t-tests to compare sexes. An exploratory principal components analysis (PCA) was conducted (JMP®, Version 14. SAS Institute Inc., Cary, NC, 1989–2019) to visualize the parameters most associated with determining sex. MANOVA was performed on all variables. A recursive partitioning tree was generated using the R package "*rpart*" along with a linear discriminant analysis of the data using the R package "*MASS*" to produce a decision tree with cut-off values for diagnostic measurements. Data were scaled to have an equal variance of 1 using the R scale function for both linear discriminate analyses and recursive partitioning (Dykstra *et al.* 2012).

Ethics Statement

This project was approved by the University of Otago's Animal Ethics Committee (#AUP D69/17) and Marshall University Office of Research Integrity's Institutional Animal Care and Use Committee (IACUC) under protocol #686. All field work and permissions were granted under Department of Conservation (DOC) permit authorisation number 38882-RES. Samples were exported from New Zealand under DOC export authorisation number 61143-DOA and imported to the United States under USFWS import permits MA69220C-0 (2018) and MA16573D-0 (2019) and USDA import permit 104291.

RESULTS

All morphological characters differed significantly between sexes against a Bonferroni corrected = 0.01 (MANOVA, $F = 29.396$, Wilks $\lambda = 0.19587$, $p < 0.001$; Table 1) with males being larger than females in each measurement, but mass was the least significant ($p < 0.01$, variable importance = 13; Figure 2). PCA indicated separation by sex when all variables were considered with PC1 reflecting overall size and explaining 63.2% of the variation (Figure 3). Recursive partitioning of the data indicated cut-off values to classify tawaki sex and the resulting decision tree identified foot length as the most distinguishing variable (Figure 4). A linear discriminate analysis indicated that the morphological parameters correctly classified 94% of the penguins sampled (95% males, 93% females).

Table 1. Morphological parameters assessed in 34 (13 male; 19 female) tawaki (Fiordland crested penguins *Eudyptes pachyrhynchus*). Mean values, standard deviation, and statistics for each metric assessed. All were found to be significant following MANOVA and Bonferroni correction of $\alpha = 0.01$. All metrics other than mass were significant at $p < 0.001$.

Measurement	Male	Female	*F*	P (< 0.01)
Mass (kg)	3.12 ± 0.40	2.69 ± 0.29	12.056	< 0.01
Bill Length (mm)	48 ± 2	43 ± 2	45.937	< 0.001
Bill Depth (mm)	28 ± 3	23 ± 3	38.955	< 0.001
Head Length (mm)	125 ± 4	116 ± 6	26.396	< 0.001
Foot Length (mm)	117 ± 6	108 ± 3	31.016	< 0.001

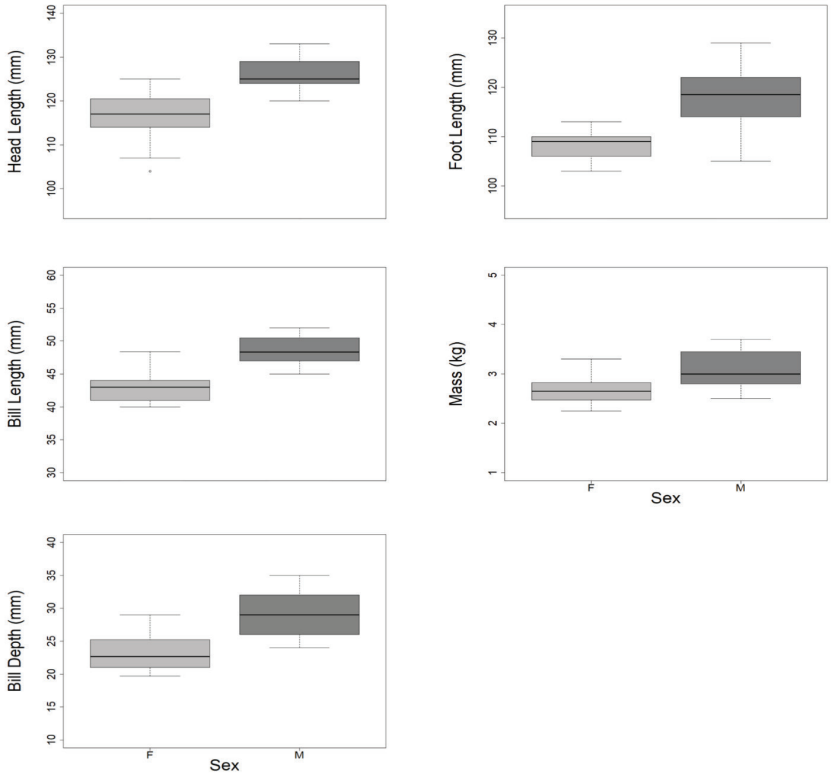

Figure 2. Morphological parameters measured in tawaki (Fiordland crested penguin *Eudyptes pachyrhynchus*) showing median measurement (black bar), interquartile range containing 50% of the data (shaded region), the range with upper and lower 25% of data (grey lines), minimum and maximum values (grey bars), and any outliers (individual points). While males were generally larger than females in all measurements, bill depth, bill length, head length, and foot length showed the least overlap.

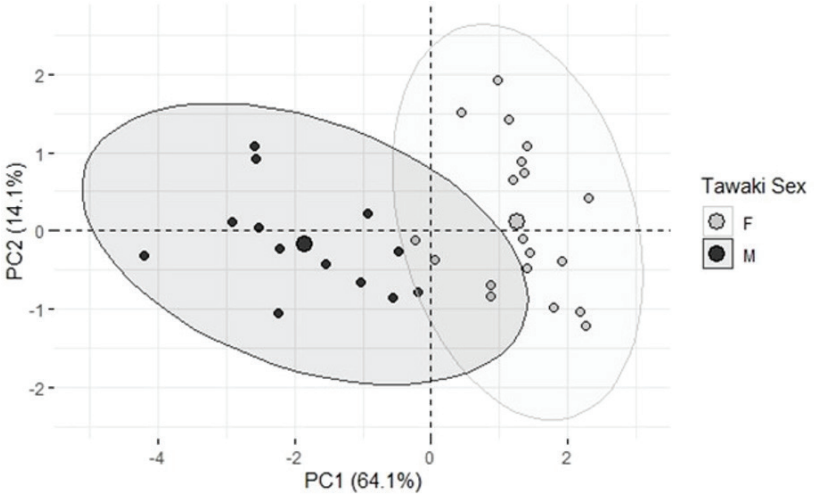

Figure 3. Principle components analysis (PCA) of morphological parameters examined in tawaki (Fiordland crested penguin *Eudyptes pachyrhynchus*). PC1 explained 64.1% of the variation between the sexes while PC2 accounted for 14.1%. All but one male fell outside of the confidence ellipse for females, but four females overlapped within the male confidence ellipse.

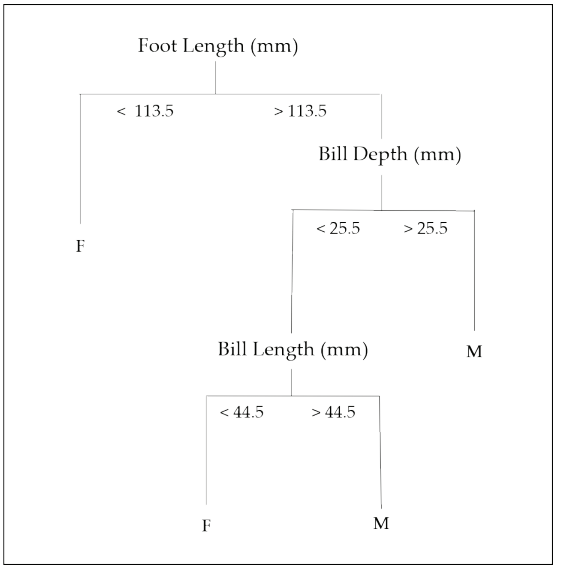

Figure 4. Recursive partitioning tree of foot length, bill depth, and bill length; 94% of tawaki (Fiordland crested penguins *Eudyptes pachyrhynchus*) were correctly classified by following these parameters.

DISCUSSION

Accurately determining sex of tawaki in the field is integral to ecological, behavioural, and demographic research and conservation efforts. Like other crested penguins, tawaki are not visually sexually dimorphic making reliable field sexing challenging outside incubation and guard stages. Although tawaki have predictable sex-specific roles during these periods, they behave more similarly at other times of the year (Warham 1974). During the post-guard period, behaviour alone is inadequate as male tawaki leave their nest sites when chicks form crèches in the forest. Additionally, non- breeding individuals are present in the colony and moulting tawaki cannot be sexed using breeding period specific behaviour.

We favoured parameters that can be measured quickly in conjunction with other sampling procedures. Mass was shown to be the least significant variable measured, which was expected given the life stage examined in the study period. From late incubation through the guard stage male tawaki fast while females forage daily. This foraging difference potentially reduces the disparity in mass between sexes when compared to other periods of the annual cycle. Therefore, we do not recommend using mass as a factor in sex determination as it is dependent on overall body condition.

Linear measurements of skeletal size exhibited greater variation between the sexes ($p < 0.01$ for all). Warham (1972) and Murie *et al.* (1991) supported the bill shape index to sex tawaki. Our data also indicated that measurements associated with bill and head size (bill length, depth, and head length) were significantly larger in males. We suggest using foot length (males > 113.5 mm) in conjunction with bill length (males > 44.5 mm) or bill depth (males > 25.5 mm) as the most reliable metrics to identify the sex of adult tawaki in the field.

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