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Bill Shape Variation in African Penguin (*Spheniscus demersus*) Held Captive in Two Zoos

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Abstract: Penguins, like most birds, are considered monomorphic species. Cloacal endoscopy, laparoscopy, or molecular sex verification are used to determine sex in such animals. Our aim in this study was to investigate whether sex recognition can be performed in penguins by a non-invasive method using the shape of the bill. For this purpose, sex and population differences in penguins were investigated by geometric morphometric methods using photos of the bill in the dorsal and lateral views. Fifty-four African penguins (*Spheniscus demersus*) were taken for the study. Principal component analysis was applied to reveal the shape variations of the bill. Principal components were extracted for each bill projection. PC1 explained 37.06% of the total variation in the dorsal view, while PC1 for the lateral view explained 31.4% of the total variation. Canonical variance analysis was performed to reveal the differences between groups. The lateral view was more effective in revealing the differences between the groups and between the sexes. For the dorsal view, Procrustes distances values between any group were not statistically significant. The maxillary rostrum in female penguins was higher, while, in males, the mandibular rostrum was higher. The females' bills were narrower than in males. Centroid size in males was on average larger than in females. Significant differences in bill shape between populations were also found. Using geometric morphometric methods, sex analysis can be conducted with less equipment and less stress on the birds. However, the environmental factors that cause bill variation in birds should be examined in more detail. Better knowledge of the effects of environmental factors on bill variation is important for geometric morphometric methods to give more accurate results in sex and population analyses.

Keywords: geometric morphometry; bill variations; sex differences

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1. Introduction

The African penguin (*Spheniscus demersus*) is endemic to coastal areas of Southern Africa [1]. The population of the African penguin continues to decline, and, since 2010, it has been declared an endangered species [2]. Like other penguin species, these birds cannot fly. Their wings are stiff and flattened as an adaptation to marine habitats. Endangered African penguins are a common species in zoo gardens around the world [3].

Traditional methods of determining the sex of certain species, such as the *Spheniscus* genus, have relied on techniques like biometric measurements, observation of the copulatory behavior, cloacal examination, or dissection [4]. However, molecular techniques

have emerged as a promising approach for accurately identifying the sex of individuals at any life stage. The most employed method in avian sexing involves polymerase chain reaction (PCR) and analysis of the chromo-helicase-DNA-binding (CHD) gene found on the W and Z chromosomes. The CHD-W gene is specific to females, while the CHD-Z gene is present in both males and females. Therefore, using the difference in the intron lengths of the CHD-W and CHD-Z genes yields two fragments for females (W, Z) and only one fragment for males (Z, Z) [5].

Geometric morphometrics (GM) is a method that analyzes all geometric information taken from the Cartesian coordinates of anatomical points [6]. Landmarks are placed on 2D or 3D samples to perform the analysis. Then, using these landmarks, the shape is obtained for each sample. The samples' shape variations can be obtained using principal component analysis (PCA) [7]. Various principal components are obtained with PCA. The difference between paired groups can be obtained statistically by using the discriminant function [8,9]. In analyses such as the discriminant function, the average shape of the groups is obtained. The results are interpreted statistically by obtaining the Procrustes distances of the average shapes between the groups. Various studies comparing the morphological features of animals using these features of GM are included in the literature. For example, in studies on skulls, shape variations among species in the same family have been revealed [10]. In addition, studies using shape analysis have investigated whether there is a sex difference [11,12].

Most bird species are monomorphic taking into account plumage, appearance, behavior, or morphological features [13]. Therefore, various methods are used for sexing in birds. Cloacal endoscopy and laparoscopy are relatively effective, but these methods are invasive applications that cause stress to animals [14]. In recent years, linear bill measurements in birds have been shown to determine sex, and this method has been applied to a variety of bird species [15]. In this study, it was investigated whether the bill shape of the African penguin is dimorphic using GM. In an attempt to reveal differences between the sexes in the two different populations, bill shape was studied using photographs in dorsal and lateral projections.

2. Materials and Methods

2.1. Animals

A total of 54 (32 female; 22 male) African penguins were used in our study. Specimens were collected from two different zoo gardens in Turkey: Faruk Yalcin Zoo (n: 31) and Bursa Zoo (n: 23). Faruk Yalcin Zoo is located in Darica, in Kocaeli Province. Bursa Zoo is located in Bursa Province. The penguins used for the study were adults. The animals were healthy, in good condition, and with no pathological disorders. Before the samples were collected, clinical examinations were performed by specialists.

2.2. Molecular Sex Verification

The genomic DNA was purified from one to two feather calamus ends (2 mm) of each penguin using the Genomic DNA from Tissue Kit (Macherey-Nagel GmbH & Co. KG, Dueren, Germany), following the manufacturer's protocols. The CHD-W and CHD-Z gene regions were amplified by PCR from the genomic DNA using the 2550F and 2718R primers [16]. PCR amplifications were performed in 25 µL reaction mixture, which contained: 1 × Taq Buffer (Thermo Fisher Scientific, Waltham, MA, USA), 1 mM MgCl2, dNTP (0.2 mM of each), 0.5 µM of each primer, 0.5 U Taq polymerase (Thermo Fisher Scientific), and 1–5 ng of genomic DNA. Amplifications were conducted in a t100 biorad thermal cycler under the following conditions: 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 50 °C for 30 s, and 68 °C for 45 s, and a final extension step at 68 °C for 5 min. PCR products were run on 2% agarose gel for 45 min at 100 V in TAE buffer (Figure 1).

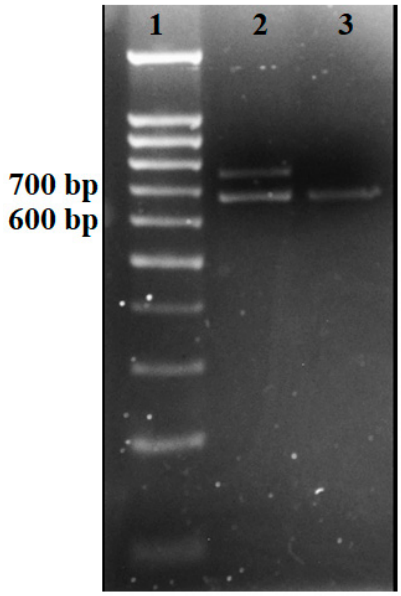


Figure 1. Identification of sex in S. demersus with 2550F–2718R primers. Lane 1: 100 bp DNA ladder (Intronbio). Lane 2: female. Lane 3: male.

2.3. Geometric Morphometry

Photographs of the birds' bills were captured in dorsal and lateral projections. The photos were taken by the same person with the same camera (Canon 500D). Images were taken at right angles from a constant distance of 15 cm. It was clarified that the photos were taken at right angles to the object. However, the exposure conditions were not standardized as they did not affect the shape of the beak. Auto exposure mode was used. The captured images were digitized for further analysis. Firstly, a tps file was created for landmark operations. For this purpose, the tpsUtil software (version 1.74) was used. Two separate files were created for both dorsal and lateral images. Then, the TpsDig program (version 2.32) was used to digitize landmarks (Figure 2). Curves containing 23 semi-landmarks were applied for dorsal images, and curves containing 53 semi-landmarks were applied for lateral images. The tps files containing the latest curve coordinates were prepared for analysis by using the "append tps curve to landmarks" option in the tpsUtil (version 1.74) program.

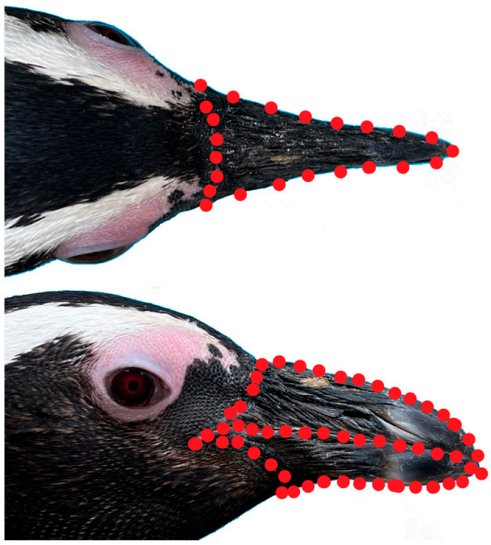


Figure 2. Landmarks.

2.4. Statistical Analysis

For the statistical analysis, the MorphoJ software (version 1.07) was used. For this purpose, the landmarks were superimposed by applying "Procrustes fit". Then, generalized Procrustes analysis was performed to render the data suitable for further statistical analysis. Generalized Procrustes analysis used different pooled within-group covariances for both sexes and populations. Shape variations were obtained by applying principal component analysis (PCA) for each statistical variable (sex and population). Principal components (PCs) were extracted as a result of PCA. Graphs were generated with PC values describing the largest shape variation. Average shape and PC changes were also shown on wire-frame warp plots. Canonical variance analysis (CVA) was used to examine the distinctions between binary groups (female–male; Bursa–Darica). A permutation test was used in the CVA analysis to assess the statistical significance of the relationship between sets of variables from different sexes and populations. As a result of CVA, intergroup Procrustes distances values were obtained. *p* values were obtained by comparing each group. Results with a *p*-value below 0.05 were considered statistically significant. Permutation runs were analyzed as 1000. The mean centroid size for each group was estimated.

3. Results

Principal component analysis was performed to reveal shape variations among samples. As a result of the PCA test, 21 PCs were found for the dorsal view of the bill and 53 PCs for the lateral view. PC1 explained 37.06% of the total variation in the dorsal view, while PC1 for the lateral view explained 31.4% of the total variation. PC results are given in Table 1.

Table 1. PCA results.

Dorsal			Lateral		
PC	**Eigenvalues**	**%Variance**	**PC**	**Eigenvalues**	**%Variance**
PC1	0.00161037	37.063	PC1	0.00226000	31.404
PC2	0.00094577	21.767	PC2	0.00125549	17.446
PC3	0.00077402	17.814	PC3	0.00095716	13.300

In Figure 3, the distribution plots of the samples according to PC1 and PC2 are given for both the dorsal and lateral views. The first two components explained 58.83% of the total variation for the dorsal view; 48.85% of the total variation was explained by PC1 and PC2 in the lateral view. In both analyses, it was observed that the sex or population distribution did not differentiate as shape variation. For the dorsal view, PC2 values were generally negative, except for three individuals in the Darica female samples. Bursa samples were mostly found to have high PC1 values for the lateral view.

Wire-frame warp plots of changes in the bills are given in Figure 4. In the wire-frame warp plots, the blue lines represent the average shape. The red color is responsible for the positive limit of the PC value. This means that the figure shows a change from blue to red as the PC value from 0 to a positive value. When the PC value changes from 0 to negative, the sample shows a shape variation from blue to the inverse of red. According to the dorsal view results, the change in PC1 was seen most distinctly on the posterior part of the bill. An increased PC1 value represented a wider bill. In addition, with an increasing PC1 value, the lateral borders of the bill extended more caudally, while the base of the bill in the middle was shifted more rostrally. With the increasing PC2 value of the dorsal aspect, the bill was shorter but wider. As for the lateral view results, the change in PC1 was most pronounced in the height of the bill. With an increasing PC2 value, the height of the bill was below the average shape. In the lateral view, the maxillary rostrum was shorter with an increase in both PC1 and PC2 values.

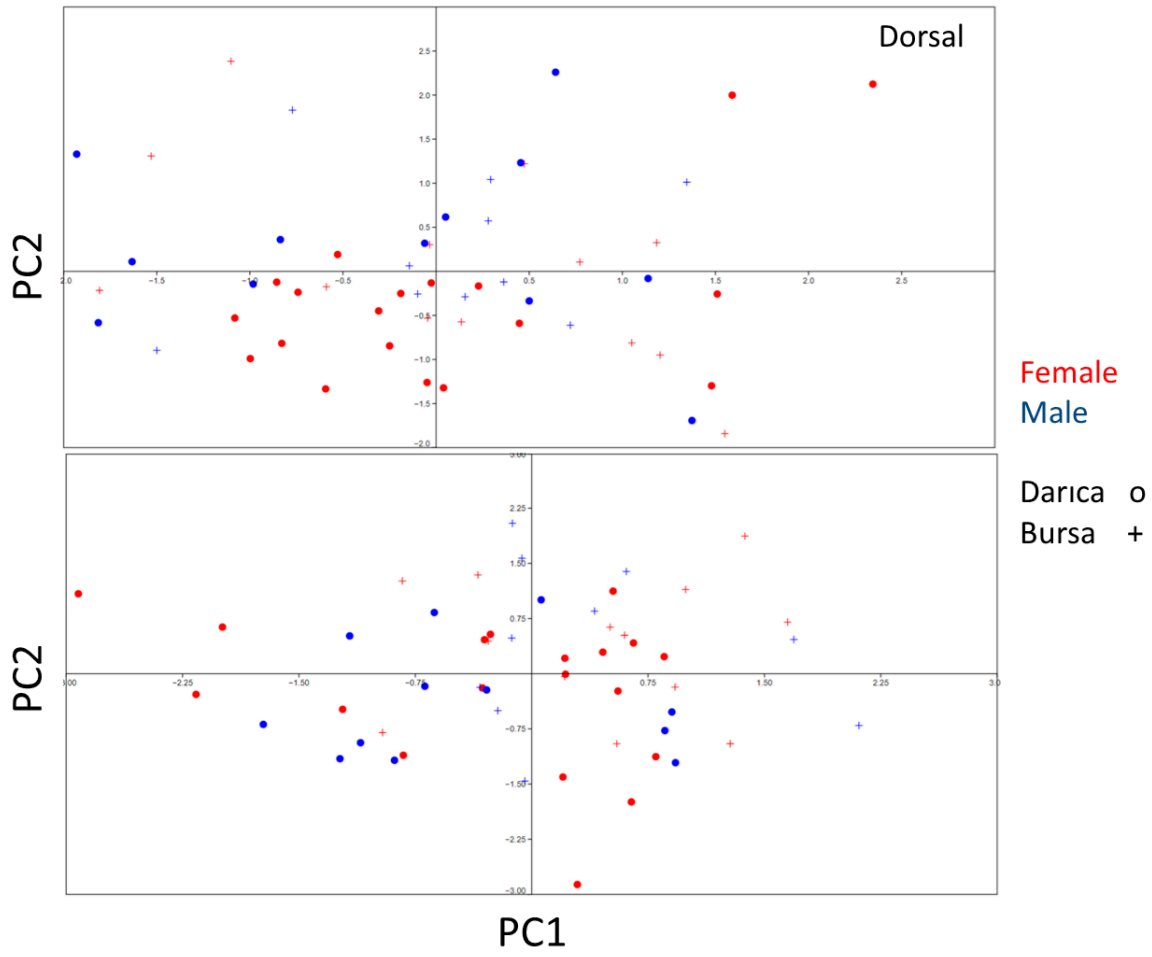


Figure 3. Scatterplots of PC1 and PC2 of bill shape in dorsal and lateral view for sexes and populations.

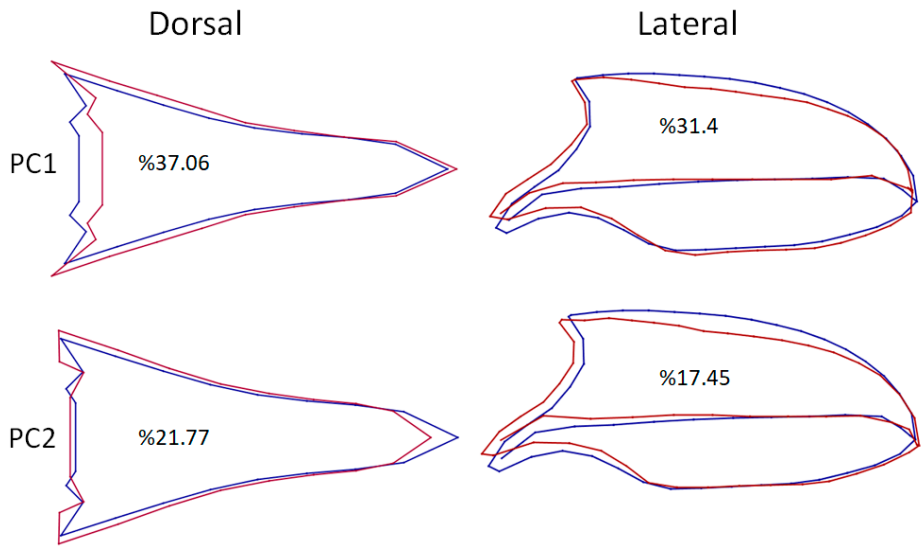


Figure 4. Wire-frame warp plots of shape changes in the bills. Blue lines represent the initial shape and red lines represent shape changes in the direction of PC1 and PC2 in their positive limits.

Scatterplots of CV1 and CV2 of the bill shape in the dorsal and lateral view for sexes and populations are given in Figure 5. In general, the clear separation of individual groups was observed in the lateral view. For the dorsal view, males had negative CV1 and females had positive CV1. However, all groups overlapped to a large extent within the

morphospace. In the lateral view, it was observed that all groups were separated from each other except for one specimen (Darica, male). Bursa samples had a positive CV1 value for the lateral view, while Darica samples had a negative CV1 value (except for one sample in each of the two populations). Wire-frame warp plots of changes in the bills for CVA are given in Figure 6. The increased CV1 value for the dorsal view represented a narrower bill. Females with a higher CV1 value than males had a narrower bill than males. The maxillary rostrum was narrower with a positive value of CV1 for the lateral view. According to these results, while the maxillary rostrum in female penguins was higher, the mandibular rostrum in male penguins was higher.

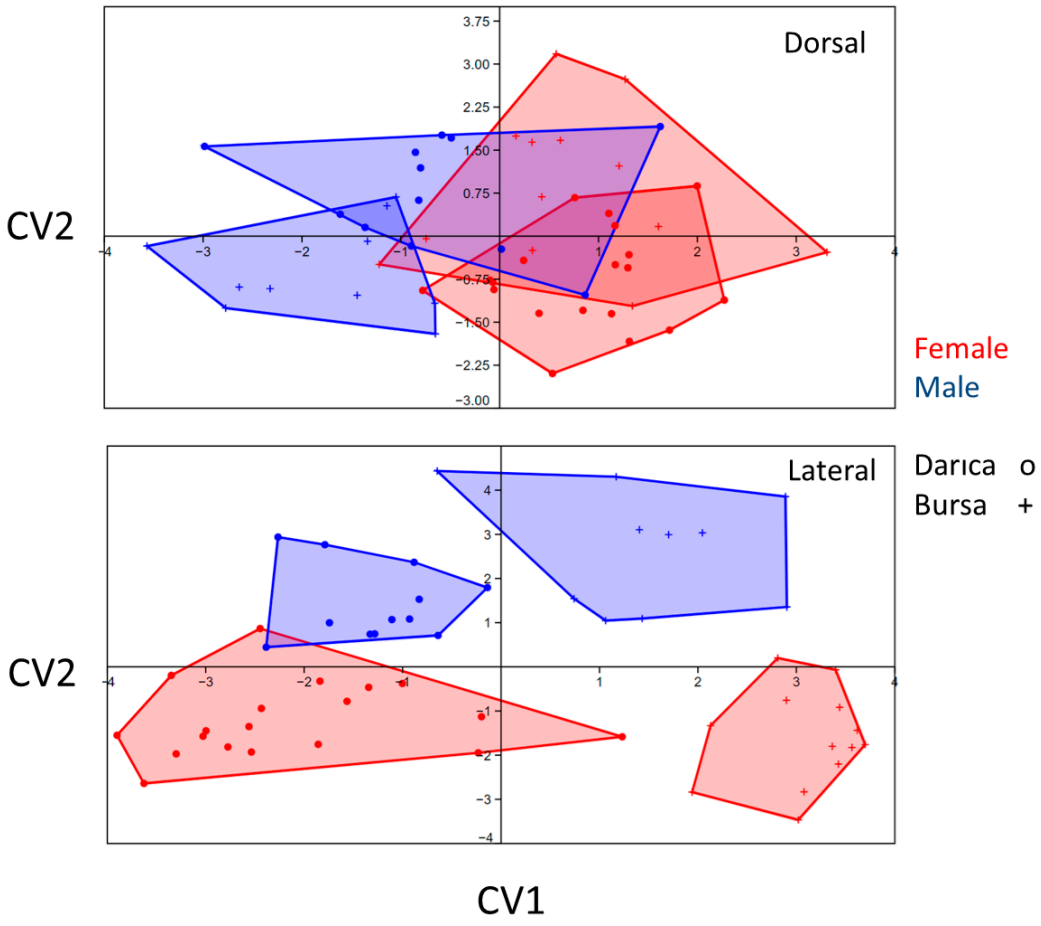


Figure 5. Scatterplot of CV1 and CV2 of bill shape in dorsal and lateral view for sexes and populations.

Procrustes distances values are given in Table 2. According to these values, statistically significant differences were found in the lateral view. According to analyses from the dorsal view, the differences between groups were statistically insignificant. The greatest difference was revealed between males from Darica samples and females from Bursa samples in the lateral view (p: 0.0177). The next most significant difference was between male Bursa samples and male Darica samples for the lateral view (p: 0.0220). For the dorsal view, the Procrustes distances values between any group were not statistically significant.

The distribution of the dorsal and lateral analysis results of the samples according to sex or regional differences is given in Figure 7. Disregarding regional differences, the lateral appearance was found to be discriminatory for the sexes (one female was misclassified). After ignoring sex differences, the lateral view was found to be characteristic in terms of population (one Darica and one Bursa sample were misclassified).

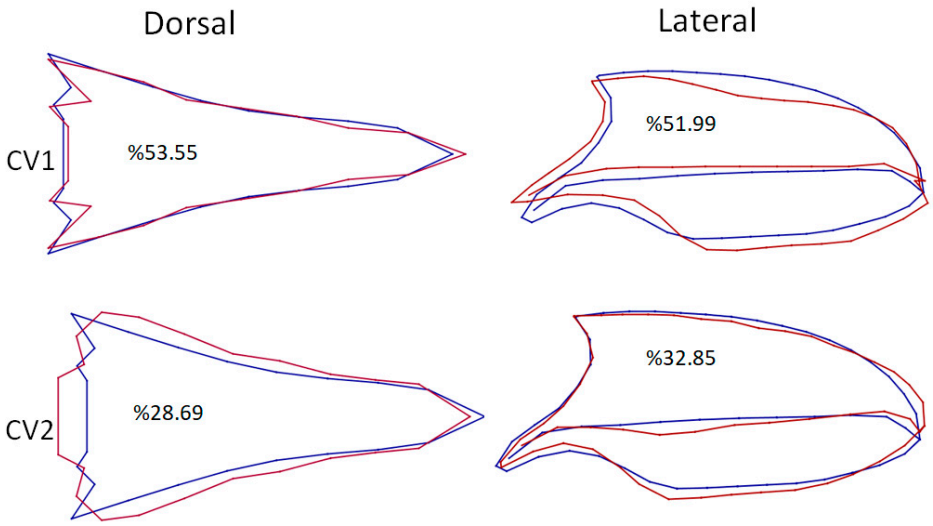


Figure 6. Wire-frame warp plots of changes in the bills. Blue lines represent the initial shape and red lines represent shape changes in the direction of CV1 and CV2 in their positive limits.

Table 2. Procrustes distances values and *p* values.

	Female–Bursa		**Female–Darica**		**Male–Bursa**	
	P-D	***p* Value**	**P-D**	***p* Value**	**P-D**	***p* Value**
Female–Darica	0.0185/0.0444	0.7001/**0.0499**				
Male–Bursa	0.0179/0.0272	0.8396/0.7951	0.0215/0.0486	0.706/**0.0387**		
Male–Darica	0.0200/0.0537	0.7619/**0.0177**	0.0281/0.0262	0.2695/0.6344	0.0231/0.0542	0.6484/**0.0220**

P-D: Procrustes distance; dorsal/lateral; bold: statistically significant.

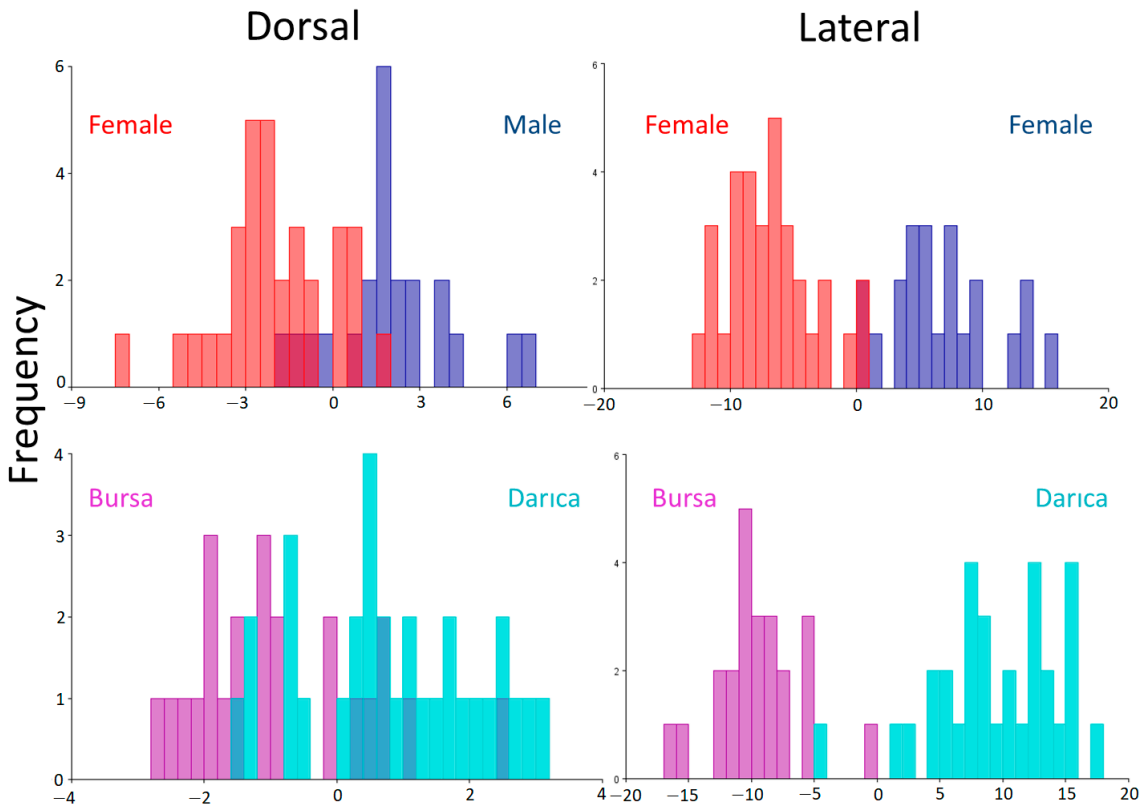


Figure 7. Distribution of samples by dorsal and lateral analysis according to sex or population differences.

The centroid size and standard deviations by sex and population are given in Table 3. According to the results, it was seen that the centroid size was statistically different in the lateral view. In this view, the centroid size of the bill in males was larger than that in females. In addition, the centroid size of penguins from the Bursa Zoo garden was higher than that of Darica in the lateral view. These differences were statistically significant.

Table 3. Centroid sizes and standard deviations.

	Sex	n	Mean	Sd	*p*-Value
Dorsal	Female	32	1.77591×10^{16}	1.61886×10^{16}	0.42
	Male	22	2.17361×10^{16}	1.9654×10^{16}	
Lateral	Female	32	9.10566×10^{15}	9.48929×10^{15}	**0.01**
	Male	22	1.65972×10^{16}	1.14494×10^{16}	
Dorsal	Bursa	23	1.50812×10^{16}	1.62389×10^{16}	0.12
	Darica	31	2.25684×10^{16}	1.81664×10^{16}	
Lateral	Bursa	23	1.7753×10^{16}	1.37855×10^{16}	**0.01**
	Darica	31	8.00648×10^{16}	5.29417×10^{15}	

Bold values denote statistical significance at the $p < 0.05$ level.

4. Discussion

In this study, we sought to find differences in bill shape between male and female African penguins belonging to two populations residing in two zoo gardens located in Western Turkey. We used this body part because of its easy accessibility regardless of the imaging method. Differentiation between the sexes of birds based on the shape of the bill has been described by many authors [17–20]. This also applies to penguins [21–23], including the African penguin [4]. No sex-related differences in bill shape have been noticed by other authors [24]. As reported by many researchers, sexual dimorphism in birds is expressed more in differences in size than in external morphological features or the shapes of individual body parts [20,25,26]. In most birds, males are larger than females, but sometimes the opposite is true [27,28]. The phenomenon of allometry makes it difficult to differentiate sexes, especially if we compare individuals of different ages and physiological conditions.

Geometric morphometrics allows us to compare shapes regardless of scale, rotation, and translation. On the other hand, this method is able to capture subtle differences in shape, inaccessible to traditional linear morphometry [6]. Therefore, in our work, we used geometric morphometry to assess the sources of bill shape variability in African penguins kept in captivity. Principal component analysis allowed for the extraction of principal components, the first three of which captured 76.64% percent of the total variance in the bill shape for the dorsal view and 62.15% for the lateral view. Canonical analysis of variance showed that the bill shape variation in the African penguin in our study was mainly due to belonging to different populations rather than sexual dimorphism. The greatest variability in this respect occurred in the lateral projection of the bill. According to Campbell et al. [4], the sex-differentiating feature of the African penguin is primarily the size of the beak. Individual measurements overlap between males and females. The discriminant function proposed by the authors is based on the length and depth of the beak. It has been shown to be 90% effective in cross-validation. These conclusions are consistent with our results, which showed significant differences in beak shape only in the lateral view. The shape variation of the bill can be explained through a combination of genetic variation, natural selection, and environmental factors [29–33]. It has been reported that the bill sizes of the birds are variable in different geographical regions. Symonds [34] proved that there was strong evidence of bill lengths being shorter at colder temperatures (lower Tmin) within Australian parrots, Canadian galliforms, penguins, and gulls in his study on birds. Due to the relatively small geographical distance between the captive populations in our study, it can be assumed that climatic conditions were not significant in this case.

Bill variations in different Chukar Partridge populations were studied using geometric morphometrics [24]. In this study, although there was a size difference between the sexes, no difference in shape was found. However, differences in shape were observed between populations. A similar result to the previous study was obtained in this study in penguins. The bill size in male penguins was larger than that in females. However, this difference was statistically significant only in images in the lateral view. Likewise, shape variations in the lateral view were statistically different between penguin populations.

Greenberg et al. [35] studied the effect of different temperatures on bill size. They stated that the bill surface area increases with the summer temperature as a result of this work. Peterson [36] examined bill shapes in different geographic regions in a study conducted on scrub jays. He reported that populations living in oak woodlands had short, hooked bills, whereas populations living in pinyon–juniper woodlands had long, pointed bills. In this study on penguins, samples from two different populations kept in captivity in zoo gardens located in Western Turkey were examined. However, the living conditions of the animals in the study were similar. In addition, the geographical features of the two zoos were very close to each other. However, differences in bill shape variations were found between the two populations. These differences can be considered to be related to physiological or social factors. Dietary habits can also affect bill shape variations. According to previous studies, Spheniscus is the most piscivorous of all the living penguins, feeding on fishes and squids. However, we did not have information to explain the results of the study and the main reason for bill shape variations in terms of physiological or nutritional habits. This hypothesis may be the aim of our next study. Wallace et al. [37] noted that the beak shape and size in Humbolt penguins differed significantly between captive and free-living individuals. According to the authors, birds kept in captivity are characterized by the overgrowth of the beak. A similar phenomenon has been observed in other bird species [38]. Moreover, in Campbell's research [4], African penguins from geographically diverse colonies differed in beak size. The authors noted larger dimensions in captive individuals compared to wild ones. This means that the results obtained on captive populations cannot be transferred to free-living individuals and vice versa. Our results show that two captive populations isolated from each other, without the possibility of mutual gene exchange, differed from each other and these differences were greater than related to sex. It would therefore be worthwhile to perform similar studies on wild African penguins.

5. Conclusions

In the study, bill shape variations in African penguins belonging to two different populations kept captive were examined. Males had larger bills than females. The sizes of the samples belonging to Faruk Yalcin Zoo (Darıca) were also greater than those from Bursa Zoo. However, this difference was statistically insignificant in the dorsal view. The difference in the lateral view was more pronounced and statistically significant. In terms of shape, the difference in the lateral view was more pronounced between the groups. Using geometric morphometric methods, sex determination can be performed with quite a simple, low-cost, and non-invasive method, without unnecessary stress for birds. However, environmental factors that cause bill variation in birds should be examined in more detail. Better knowledge of the effects of environmental factors on bill variation is important for geometric morphometric methods to give more accurate results in sex and population analyses.

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