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Sex determination by morphometry of adult White-crested Elaenia (*Elaenia albiceps chilensis*)

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ABSTRACT: White-crested Elaenia (*Elaenia albiceps chilensis*) is a Neotropical austral migrant that is sexually monomorphic in plumage. We first determined whether the brood patch and cloacal protuberance are good indicators of an individual's sex by comparing these characters with individuals sexed using molecular techniques. Second, at several localities encompassing a 1500 km latitudinal gradient across the breeding range, we evaluated whether morphological measurements can be used for sex determination, using discriminant analysis and molecular sexing as dependent variables. Finally, the effectiveness of the discriminant analysis was assessed by a Jackknifed validation, and by a cross-validation process through the classification of a new sample. Sexing using genetic techniques and by cloacal protuberance size and presence of the brood patch produced the same results. We did not find differences in body measurements among study sites. In all localities, males were significantly larger than females in wing and tail lengths, whereas other variables did not exhibit differences between sexes. The best classification of sex by discriminant functions was obtained by including wing and tail length as discriminatory variables. The discriminant function correctly determined the sex of 86 % of all samples, with correct identification of 90 % of males and 75 % of females. The Jackknifed validation and cross-validation of a new sample resulted in similar sex classifications as those produced using the discriminant function. Discriminant analysis thus represented a simple and cost-efficient way to determine the sex of White-crested Elaenias for field ornithologists.

KEY-WORDS: Discriminat analysis, latitudinal gradients, Neotropical austral migrant, sexual size dimorphism.

INTRODUCTION

Sex determination of birds is key to understanding various aspects of the demography of a population as it may influence survival, dispersal, recruitment and other life history parameters (Gowaty 1993, Newton 1998), but sex differentiation is difficult in species with sexually monomorphic plumage, even for captured individuals. During the breeding season, the sex of most individuals of many species can be determined either by the presence or extent of a brood patch in females or the cloacal protuberance in males (Ralph *et al.* 1993). In some species, however, the male can develop an incubation patch, but it does not typically develop as extensively as in females (Skutch 1957, Pyle 1997). Furthermore, during the non-breeding season these characters are not useful, although sexing birds in the non-breeding season is desirable (e.g., sex-specific migration phenologies, migration routes, and wintering habitat use).

Morgan (2005) discussed the possibility of analyzing multi-modal distributions of morphological characters to differentiate sexes. This approach was used by Catry *et al.* (2005), who separated male and female Chiffchaffs (*Phylloscopus collybita*) based on the bi-modal distribution of wing length. However, although this method is based on sound assumptions, there is no independent verification of its reliability. Relatively unproblematic and reliable is taking a blood or tissue sample for later molecular sex determination in the laboratory (Griffiths *et al.* 1998). Many recent authors have identified useful morphological measurements for sex determination using molecular methods on a sub-sample of the species under study (Hipkiss 2007, Ottvall & Gunnarsson 2007). The sex is treated as a dependent variable in either a discriminant analysis or a logistic regression with a number of morphological measurements as explanatory variables. In recent years, these methods have been used to investigate morphological variables useful for sexing a number of non-passerine and passerine bird species (e.g., Campos *et al.* 2005, Svagelj & Quintana 2007, Cardoni *et al.* 2009).

Although these methods can lead to reliable sex determination of the population under study, their general application has some caveats (Ellrich *et al.* 2010). For

example, statistical models are selected to fit a particular sample, and will therefore fit the particular sample better than they would fit the entire population from which the sample was drawn, or a sample from another population. Also, there are intra-specific differences in morphology, such as wing length, due to different migration distances (e.g., Lindström *et al.* 1996, Fiedler 2005).

White-crested Elaenia (*Elaenia albiceps chilensis*) is a Neotropical austral migrant and is abundant in *Nothofagus* forests of Patagonia between October and March (Grigera *et al.* 1994). This species is a small tyrant flycatcher (Tyrannidae) sexually monomorphic in plumage (Fitzpatrick 2004). There is little research about the White-crested Elaenia demography, which is hampered by the lack of data enabling researchers to determine sex in the field. Our aim was to evaluate sex determination of White-crested Elaenia using molecular techniques and morphometric measurements. First, we analyzed whether the presence of a brood patch or cloacal protuberance are good indicators of an individual's sex in known-sex individuals as determined through molecular techniques. Second, at several localities encompassing a 1500 km latitudinal gradient of the breeding range of White-crested Elaenia, we evaluated whether morphological measurements can be used for sex determination, using discriminant analysis and molecular sexing as dependent variables. Third, we assessed the effectiveness of the discriminant analysis in terms of the proportions of individuals of known sex that were classified correctly by a Jackknifed validation, and by cross-validation through the classification of a new sample.

METHODS

Study sites

Between October and March, White-crested Elaenias mainly inhabit *Nothofagus* forest within a narrow strip approximately 2000 km long, extending from the southern tip of South America to northern Patagonia. Along this latitudinal gradient, we selected four study sites in National Parks of Argentina: Parque Nacional (PN) Tierra del Fuego (54°49' S, 68°28' W), Province of Tierra del Fuego; PN Los Glaciares (49°14' S, 72°54' W), Province of Santa Cruz; PN Perito Moreno (47°57' S, 72°07' W), Province of Santa Cruz; and PN Los Alerces (42°36' S, 71°38' W), Province of Chubut (Figure 1). For cross-validation classification of a new sample, we selected a site near Esquel (42°55' S, 71°21' W), Province of Chubut (Figure 1). Vegetation corresponds to the Subantartic Biogeographical Province (Cabrera & Willink 1980). In the northern part of this latitudinal gradient, the climate is characterized by cold and wet winters and mild but dry summers. Most precipitation falls as rain and snow during autumn and winter. South of 55° S, precipitation is more evenly distributed throughout the year (Garibaldi *et al.* 2011).

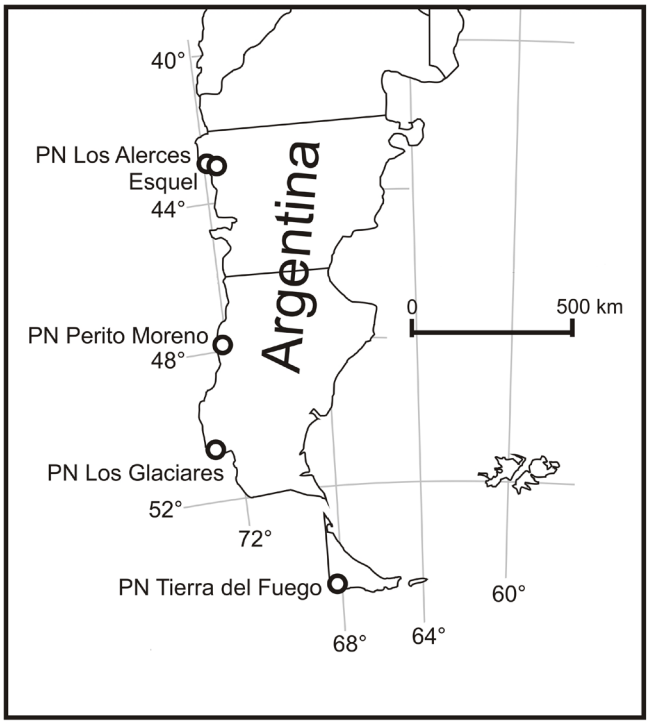

FIGURE 1. Geographical locations where White-crested Elaenias (*Elaenia albiceps chilensis*) were sampled in Patagonia, Argentina.

Sampling birds

Scientific nomenclature and common name follow the recommendation by the South American Checklist Committee (www.museum.lsu.edu/~Remsen/SACCBaseline.html, accessed on 20 May 2014). We only sampled breeding individuals to avoid the possibility of capturing individuals during migration. We sampled at the four National Park study sites from 1 January to 3 February 2012, and sampled at Esquel site from 10 December 2013 to 15 January 2014. Birds exhibiting territorial displays were captured by placing a White-crested Elaenia model coupled with conspecific songs and calls delivered through a portable speaker within 2 m of a mist net (12 x 3 m, 38-mm mesh size). At Esquel site, we passively captured birds using 10 mist nets (12 x 3 m, 38 mm mesh size) placed 70-100 m apart during four occasions for two consecutive days. Nets were opened during the first four to five hours after sunrise when weather conditions were not adverse (rainy or windy).

At all sites, captured birds were banded with numbered aluminum bands. For each captured individual, we recorded the size of the cloacal protuberance and stage of development of the brood patch. Cloacal protuberances were categorized as: none (0), small (1), medium (2), and

large (3) and brood patch development was categorized as follows: not present (0), smooth skin (1), vascularized (2), wrinkled (3), and molting (4) (Ralph *et al.* 1993). We also took five morphological measurements from each adult bird: bill length (from the anterior end of the nostril to the bill tip), tarsus length (from the intertarsal joint to the distal end of the last leg scale before the toes emerge), tail length (from the base of the feathers to the end of the longest feather), wing chord (from the carpal joint to tip of the longest primary), and body mass. We used a digital caliper (± 0.01 mm) for bill and tarsus measurements, a wing ruler (± 1 mm) for tail and wing measurements, and a digital scale (± 0.1 g) to record weight.

Molecular analysis

For molecular sexing, we collected blood samples by piercing the brachial vein with a sterile needle and sampled blood using a 0.5-ml heparinized capillary tube. Blood samples were stored in absolute ethanol at room temperature. DNA from approximately 1 mm^3 of blood was purified, according to the protocols presented in Ivanova *et al.* 2006.

For molecular sex determination, we amplified a fragment of the Chromobox-Helicase-DNA-Binding (CHD) gene by utilizing PCR with primers P2 and P8 (Griffiths *et al.* 1998). The PCR procedure used approximately 40 ng of DNA and a primer concentration of 0.5 µM. PCR reactions used an initial denaturing step of 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 45 s at 49°C, and 45 s at 72°C and a final step for 10 min at 72°C. PCR products were scored by electrophoresis in 3 % agarose gels stained with Ethidium Bromide. Amplification of the CHD gene in males produced a single band and two bands in females. All PCR product lengths were about 350 to 400 bp, which is the expected weight (Griffiths *et al*. 1998). All studied samples (n=34) were analyzed in two independent PCR assays that included male, female and negative controls.

Data analysis

For statistical analysis, we used the subset of individuals that were sexed by molecular techniques (23 males and 11 females). Two-way ANOVAs were used to evaluate if external morphology varied with sex and study site. We used the type III method to compute the sum of squares given our unbalanced design and we checked for normality and homogeneity of variance (Shaw & Mitchell-Olds 1993).

We applied a Discriminant Function Analysis (DFA) to morphological measurements taken from White-crested Elaenias of known sex. We did not use stepwise techniques to identify the set of variables included in the DFA, and instead included only variables that showed differences among sexes (see Table 1; Dechaume-Moncharmont *et al.* 2011). The performance of each variable and its combinations were evaluated with the Wilk's Lambda statistic.

The effectiveness of the discriminant analyses was assessed, first in terms of the proportion of birds of known sex that were classified correctly, second by a Jackknifed validation, and finally by a cross-validation process through the classification of a new sample (Tabachnick and Fidell 1996). The Jackknifed validation process classifies each individual case using a function obtained from the total sample, excluding the individual case to be classified (Tabachnick and Fidell 1996). The cross-validated process was used with a new set of individuals obtained during sampling at Esquel site. However, for this group, sex determination was not verified by molecular sexing. Individuals were classified by the presence of cloacal protuberance (males) or incubation patch (females).

RESULTS

All DNA samples showed one of the typical PCR band patterns that differentiate males (one band) from females (two bands). The sex of all 34 White-crested Elaenias that were assessed by size of the cloacal protuberance (males) or development of the brood patch (females) were also genetically verified.

We did not find any differences in the five body measurements among study sites (Table 1). At all sites, wing and tail lengths were significantly larger in males than in females, whereas the other variables did not show differences between sexes (Table 1, Figure 2).

The classification of sex using DFA for wing, tail and the combination of these two body measurements were in the Table 2. The best classification was obtained when including wing and tail length as discriminatory variables (Table 2). The resulting function (D_1, with an associated cut-off value of -0.385) was:

D_1 = (0.62 x wing length) + (0.07 x tail length) - 55.51

Individuals with discriminant function scores greater than the cut-off were classified as male and those with lower scores as female. This function correctly determined the sex of 86% of all samples, with correct identifications of 90% of males and 75% of females (Table 2). Individuals with a wing length of 76 mm and a tail length of 63 mm are males, and individuals with a wing and tail of 73 mm and 60 mm, respectively, are females (Figure 3). The Jackknifed classification correctly determined the sex of 65% of all samples, with the correct identification of 75% of males and 63% of females. The cross-validation provided similar classifications to those

produced by discriminant functions. The function derived from the cross-validation analysis correctly determined the sex of 76% of all samples, with the correct identification of 65% of males and 90% of females.

TABLE 1. Male and female body measurements (mean ± SD), of adult White-crested Elaenias (*Elaenia albiceps chilensis*) from four National Parks in Patagonia, Argentina. All measurements are given in mm except body mass in g. Factors of a Two-way Anova were Sex (S), Origin (O) and the interaction (SxO). Only indicated the results that were statistically significant at Type I error lest that 0.05.

	MALES			FEMALES			
Body Measurement	**n**	**Mean**	**SD**	**n**	**Mean**	**SD**	**Two-way Anova**
Wing Length							
PN Tierra del Fuego	6	75.8	2.6	4	72.8	2.2	
PN Los Glaciares	4	74.5	2.4	3	73.3	2.1	$F_{S; 1, 26} = 5.5$, $P = 0.027$
PN Perito Moreno	4	76.8	1.7	2	74.5	0.7	$F_{O; 3, 26} = 1.1$
PN Los Alerces	9	76.1	1.7	2	75.0	2.8	$F_{SxO; 3, 26} = 0.4$
ALL	23	75.9	2.1	11	73.6	2.0	
Tail Length							
PN Tierra del Fuego	5	62.6	2.1	2	60.0	1.4	
PN Los Glaciares	4	61.0	0.8	3	60.3	2.5	$F_{S; 1, 23} = 6.9$, $P = 0.015$
PN Perito Moreno	4	63.5	2.1	2	60.0	1.4	$F_{O; 3, 23} = 0.3$
PN Los Alerces	9	62.4	2.7	2	60.5	0.7	$F_{SxO; 3, 23} = 0.5$
ALL	22	62.4	2.1	9	60.2	1.5	
PN Tierra del Fuego	6	7.5	0.3	4	7.1	0.5	
PN Los Glaciares	4	7.4	0.6	3	7.6	0.4	$F_{S; 1, 26} = 1.3$
PN Perito Moreno	4	7.3	0.3	2	7.2	0.3	$F_{O; 3, 26} = 0.8$
PN Los Alerces	9	7.4	0.3	2	7.1	0.6	$F_{SxO; 3, 26} = 0.9$
ALL	23	7.4	0.3	11	7.3	0.4	
Tarsus Length							
PN Tierra del Fuego	5	20.9	1.2	2	20.3	0.3	
PN Los Glaciares	4	21.2	1.0	3	21.7	0.6	$F_{S; 1, 21} = 0.6$
PN Perito Moreno	4	21.8	0.7	2	21.3	0.1	$F_{O; 3, 21} = 0.2$
PN Los Alerces	8	21.6	0.5	2	21.7	1.8	$F_{SxO; 3, 21} = 0.6$
ALL	21	21.4	0.9	9	21.2	0.7	
Body Mass							
PN Tierra del Fuego	6	15.7	0.7	3	15.6	1.3	
PN Los Glaciares	4	15.4	1.3	3	16.8	1.9	$F_{S; 1, 25} = 0.1$
PN Perito Moreno	4	15.2	0.6	2	15.1	0.7	$F_{O; 3, 25} = 1.3$
PN Los Alerces	9	15.5	0.8	2	14.8	0.4	$F_{SxO; 3, 25} = 1.3$
ALL	23	15.5	0.8	10	15.7	1.4	

TABLE 2. Accuracy of sexing White-crested Elaenias (*Elaenia albiceps chilensis*) as percentages correctly classified using single measurements and a discriminant function D_1. All discriminant analyses were significant ($P < 0.01$).

			Accuracy (%)		
	Wilk´s lambda	**F-value**	**Males**	**Females**	**Total**
Tail Length	0.76	$F_{1, 26} = 8.23$	55	88	63
Wing Length	0.59	$F_{1, 26} = 18.40$	90	63	82
D_1	0.58	$F_{2, 25} = 8.98$	90	75	86

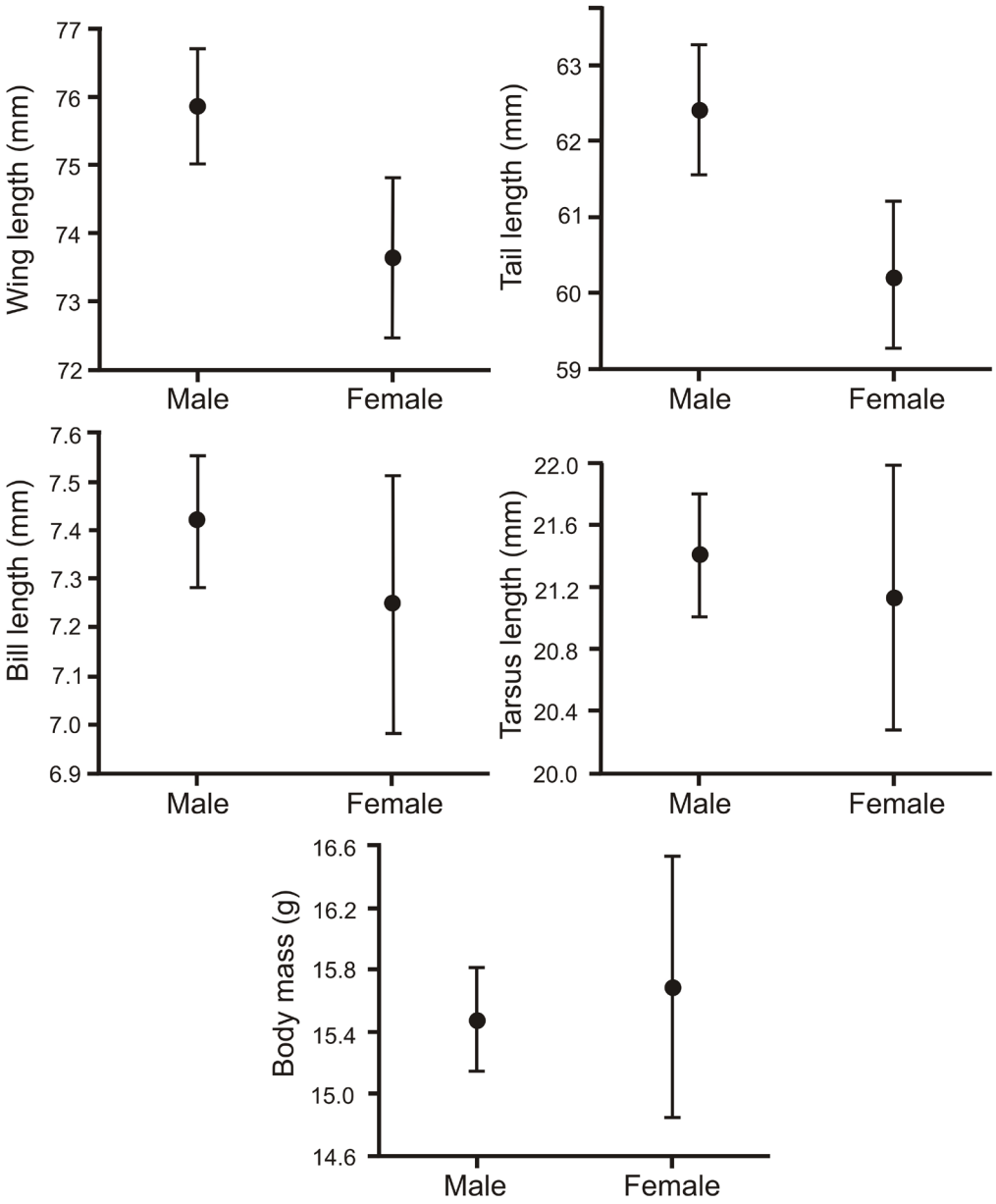

FIGURE 2. Male and female body measurements (mean ± 95% confidence interval) of adult White-crested Elaenias (*Elaenia albiceps chilensis*) of Patagonia, Argentina.

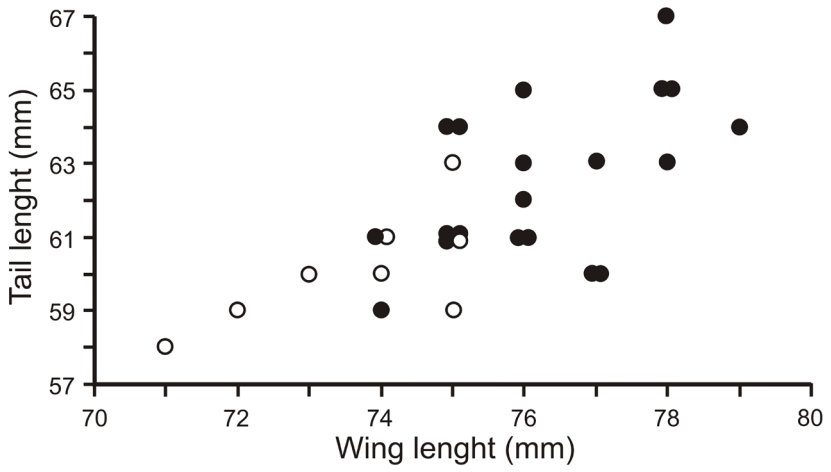

FIGURE 3. Relationship between wing length and tail length for males (black circles) and females (white circles) of adult White-crested Elaenias (*Elaenia albiceps chilensis*) of Patagonia, Argentina.

DISCUSSION

We found that White-crested Elaenias can be accurately sexed by the degree of development of the cloacal protuberance and by the presence of the incubation patch. The agreement between results of genetic sex determination and those from measurements taken from birds in the field clearly demonstrates that males do not develop a partial incubation patch. This is an important result because males in some species of flycatchers exhibit such character (e.g., in the genus *Myiarchus*, Pyle 1997). However, these features are only useful to identify the sex during the breeding season, and sexing birds in the non-breeding season is important for a proper assessment of the population dynamics of migrant birds throughout their annual cycle (Newton 2008).

Our results also show that at least two body measurements of White-crested Elaenias, wing and tail length, differ between sexes, with those of males being larger than females. Furthermore, using the discriminant function generated by the combination of these two measurements, we can differentiate the sexes with at least 90% and 75% confidence in males and females, respectively. These morphological measurements are commonly taken at banding stations and therefore the methods are highly standardized. In addition, obtaining these measurements does not require intensive handling of birds, thereby significantly reducing the stress on captured individuals.

A major criticism of the use of discriminant functions derived from body measurements to differentiate between sexes is that they are useful only for the populations from which the samples were obtained (Ellrich *et al.* 2010). In our study, we did not observe any geographic differences for the five morphological variables, even across a latitudinal gradient from 54° S (PN Tierra del Fuego) to 42° S (PN Los Alerces), i.e., separated by over 1300 km. We sampled during the active breeding period of White-crested Elaenia (corroborated by the capture of individuals in breeding condition at all sampling sites), so it is improbable that individuals captured at lower latitudes actually correspond to population that breed at higher latitudes and were migrating at the time of capture. However, we must note that for migratory birds, the wing functional features other than length *per se* could be responding to the latitudinal gradient. For example, wingtip shape is critical for speed and maneuverability in flight (Lockwood *et al.* 1998). Further studies, on the morphology and function of the wing are needed to establish whether these features in White-crested Elaenia may associate with the latitude and migration distance.

Our study shows that White-crested Elaenia males can be differentiated from females by body measurements, a common result obtained in passerines studies (e.g., Faria *et al.* 2007, Cardoni *et al.* 2009, Botero-Delgadillo 2010, Sandoval & Mennill 2013). Nevertheless, it would be important to assess other features that have been used to differentiate sexes and that can be measured during bird handling in banding stations, such as keel length. Murphy (2007) found that such a morphological measurement is very useful to differentiate the sex of individuals of Eastern Kingbirds (*Tyrannus tyrannus*). Other measurements may also be useful to differentiate the sexes in monomorphic species, for example the UV reflectance of plumage (Tubaro *et al.* 2005), although this is more difficult to obtain during fieldwork.

The results of our research are important for the analysis of geographical variation in this and other species of birds, because possible morphological differences among localities could be due to variations in the sex ratio of captured individuals in those sites. Also, these results are useful to assess whether males and females differ in their demographic characteristics and habitat selection during the non-breeding season, a period of the annual cycle poorly studied in Neotropical austral migrant birds.

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