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SUBTLE SEXUAL DIMORPHISM IN THE BAY-CAPPED WREN-SPINETAIL (*SPARTONOICA MALUROIDES*; FURNARIIDAE) UNCOVERED THROUGH MOLECULAR SEX DETERMINATION

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Resumen. – Dimorfismo sexual sutil en el Espartillero enano (*Spartonoica maluroides*, Furnariidae) descubierto a través de la determinación molecular del sexo. – La familia Furnariidae consta en su mayoría de especies sexualmente monomórficas tales como el Espatillero enano (*Spartonoica maluroides*). El objetivo de este estudio fue testar el posible dimorfismo sexual en esta especie. Realizamos la determinación del sexo de 65 individuos del Espartillero enano usando técnicas moleculares. Luego realizamos un análisis de función discriminante sobre diez medidas morfológicas para seleccionar el mejor grupo de variables capaces de diferenciar los sexos. Encontramos que la longitud de la capucha roja y la cuerda del ala fueron mayores en machos que en hembras. Además, los valores de la relación longitud-profundidad del pico fueron mayores en hembras que en machos, indicando que los machos tienen el pico más robusto que las hembras. En un análisis a poteriori, la función discriminante determino correctamente el sexo en el 80 % del total de las muestras, (71 y 89% de los machos y hembras, respectivamente). Sugerimos que, en vista de estos resultados que indican dimorfismo sexual en una especie previamente descripta de no tenerlo, otras especies de Furnaridos deberían ser mejor examinadas si el dimorfismo es en realidad común, aunque sutil, en esta familia. La significancia funcional de estas diferencias necesita adicionales investigaciones.

Abstract. – The family Furnariidae comprises putatively sexually monomorphic species, such as the Bay-capped Wren-Spinetail (*Spartonoica maluroides*). The goal of this study was to test for possible dimorphism in this species. We sexed 65 individual Bay-capped Wren-spinetails using molecular techniques. We performed subsequently a stepwise discriminant function analysis (DFA) on ten morphological measurements to select the best subset of variables capable of differentiating the sexes. We found that males had a longer rufous cap and wings than females. Furthermore, the bill length-depth ratio values were higher in females than males, indicating that males had more robust bills than females. In an *a posteriori* analysis, the discriminant function correctly determined the sex of 80% of the overall samples, (71 and 89% of males and females, respectively). We suggest that, in the light of these results indicating sexual dimorphism in a species previously thought to have none, other furnariid species be better examined to determine whether sexual dimorphism is actually common, albeit subtle, in this family. The functional significance of these differences needs further exploration. *Accepted 4 June 2009.*

Key words: Bay-capped Wren-Spinetail, cryptic sexual dimorphism, sex determination, DNA analysis, grassland bird.

INTRODUCTION

The putatively monomorphic Bay-capped Wren-spinetail is a member of the ovenbird family (Furnariidae, Remsen 2003), a family generally regarded as comprising sexually monomorphic species. The overall tendency towards sexual monomorphism has been countered by only a few studies. For example, in some species of ovenbirds males may be slightly larger than females (Remsen 2003, Roper & Hutson 2003, Moreno *et al.* 2007). Moreover, notable for their lack of sexual dichromatism, thus far only a few species from this group (e.g., Ruddy Spinetail, *Synallaxis rutilans* and Chestnut-throated Spinetail, *Synallaxis cherriei*) have been described to show subtle plumage differences between sexes (Remsen 2003). Slight differences between the sexes in other characters, such as wing chord (Winker *et al.* 1994, Faria *et al.* 2007, Moreno *et al.* 2007), tarsus length (Winker *et al.* 1994), and tail length (Winker *et al.* 1994, Faria *et al.* 2007) have been documented in a few furnariid species as well. The latter fact and the overall trend in detecting cryptic and subtle sexual dimorphism in morphology (Murphy 2007), plumage (Tubaro *et al.* 2005, Eaton 2005, 2007) and songs (Roper 2005) in a number of putatively monomorphic passerine species, caused us to re-examine the assumption that Bay-capped Wren-Spinetail is truly monomorphic.

The use of multivariate statistical approaches (e.g., discriminant function analysis, DFA) has proven successful in detecting subtle sexual dimorphism in species that have otherwise been thought to be monomorphic (Wilson 1999, Donohue & Dufty 2006, Moreno *et al.* 2007, Ottvall & Gunnarsson 2007). Such an approach requires an ability to determine the sex of individuals without the use of morphological features. In threatened species, where wide-scale collecting or laparotomy is not practical, minimally invasive methods of sex determination need to be developed. In some species this can be accomplished through behavioral differences, such as copulation and courtship behaviors (Catry *et al.* 1999) or differences in vocalization (Roper 2005). Another approach is through cloacal examination (Gray & Hamer 2001). However, these techniques can be limited in their applicability, are time-expensive, and are restricted to use during the breeding season. An alternative and reliable, minimally invasive approach to discriminate sexes involves the use of molecular techniques that amplify the chromobox-helicase-DNA-binding gene (CHD-W; Griffiths & Tiwari 1995, Griffiths *et al.* 1998). Therefore, by simply capturing, bleeding, and measuring a bird at any time of the year or at any age, a molecular approach can be combined with DFA of morphological features (see Wilson 1999, Quintana *et al.* 2003, Donohue & Dufty 2006, Ottvall & Gunnarsson 2007) to detect subtle sexual dimorphism.

The Bay-capped Wren-Spinetail is an uncommon to fairly common and local inhabitant of *Spartina* marshes of southeastern South America. Because of its local distribution in a threatened habitat, the species is listed as near-threatened (Remsen 2003, IUCN 2006). As part of a project on geographic variation in the Bay-capped Wren-Spinetail , we used a combination of molecular and morphometric analyses to test for sexual dimorphism.

METHODS

Individuals of Bay-capped Wren-Spinetail were captured using mist nets during the

breeding season in three coastal saltmarshes in Argentina: Bahía Blanca (39°01'S–56°25'W), Mar Chiquita Coastal Lagoon (37°40'S–57°22'W) and Bahía Samborombón (36°22'S–56°45'W). We used samples from 65 individuals (35 adults and 30 juveniles) for molecular sex determination and measurements from the 35 adults to conduct the DFA of morphological measurements.

Molecular sex determination. Blood samples were obtained by pricking the basilica vein using 25G $^{5}/_{8}$ needles and were stored in lysis buffer (2 M Tris HCl, 0.5 M sodium, 0.01 M NaCl and EDTA, 20 % sodium dodecyl sulfate; pH 8.0). DNA was isolated from the blood samples using protocols established in the DNeasy Kit® (Qiagen, Inc., Valencia, CA, USA). Universal primers P8 (5'-CTCCCAAGGATGAGRAAYTG-3') and P2 (5'-TCTGCATCGCTAAATCCTTT-3') were used to amplify the CHD-W and CHD-Z genes located on the avian sex chromosomes. This test is based on two conserved CHD (chromo-helicase-DNA-binding) genes located on the avian sex chromosomes of most birds (Griffiths et al. 1998). The CHD-W gene is located on the W chromosome; therefore, it is unique to females. The other gene, CHD-Z, is found on the Z chromosome and therefore occurs in both sexes (female, ZW; male, ZZ). The forward primer was labeled with fluorescent phosphoramidites (HEX; Operon Technologies Inc.). PCR amplification was carried out in a total volume of 10 μL. The final reaction conditions were as follows: 2.4 μL of ddH2O, 0.1 μL of 0.1 U of AmpliTaq Gold® DNA polymerase (Applied Biosystems), 1 μL of 10 μM of both primers, 1 μL of 25 mM of $MgCl_2$, 1 μL of 10X PCR Gold Buffer (Applied Biosystems), and 1 μL of 2 mM of dNTPs and 1.5 μL of genomic DNA. The PCR profile consisted of an initial denaturing step of 7 min at 96°C, followed by 35 cycles of repeated denaturing, annealing, and extension steps at 96 °C for 60 s, 50°C for 60 s and 72°C for 60 s, followed by a final extension step at 72°C for 5 min. Then, 1 μL of PCR product was added to 9 μL of formamide/ROX solution (Applied Biosystems), electrophoresed and detected on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems). Migration was performed in a 22-cm length capillary array using POP4 polymer (Applied Biosystems) with the following parameters: 15 kV, 100 Amp, and 11mW at 60°C for 35 min. Fragment size analysis was performed using the GeneScan® and Genotyper® software (Applied Biosystems). Samples were determined to be females when two fragments were amplified (representing the two genes: CHD-Z and CHD-W) and males when a single fragment (representing the CHD-Z gene) was amplified. In order to test the reliability of this technique for sex determination in this species, we selected, as a control, breeding pairs of Bay-capped Wren-Spinetails captured at 13 nest sites with the expectation that each nest would have one adult male and one adult female.

Morphometric sex differentiation. The morphometric analysis was conducted using the following measurements of characters that have commonly been used for determination of sexual dimorphism in other species of birds (Winker *et al.* 1994, Faria *et al.* 2007, Moreno *et al.* 2007): bill length (BL) from the anterior tangent of the nostrils to the bill tip; bill width (BW) across the base of the bill under the proximal point of the nostrils; bill depth (BD) at the anterior point of the nostrils; length of rufous cap (CR); length of black on cap (CB); tarsus length (LT) as from the joint of the tibiotarsus and tarsometatarsus to the distal edge of the most distal unbroken scute overlying the middle toe; tail length (LTai) centred, from the base of the feathers to the end of the longest feather; wing chord (WC), from the

carpal joint to tip of the longest primary; and body mass (BM, to the nearest 0.1 g). In order to make the bill measurements size independent, we calculated the ratio of bill length-depth (BLD), length-width (BLW), and width-depth (BWD). We used a digital caliper (± 0.01 mm) for bill, cap, and tarsus measurements, a ruler (± 1 mm) for tail and wing measurements and a pesola (100 g) to record weight.

Statistical analyses. We used the statistical package STATISTICA to conduct a forwards step-wise DFA to the morphological measurements taken from *Spartonoica maluroides* of known sex. The performance of each variable was evaluated with the Wilk's Lambda statistic, which decreases as discriminatory power increases (Hair *et al.* 1995). The combination of measurements that best discriminates between sexes was selected, and from that we obtained a discriminant function model. A significance level of $P = 0.15$ was selected for entry into the model and variables were retained in the stepwise model at the $P = 0.05$ level of significance. The effectiveness of the discriminant analyses was assessed by a Jackknifed classification; this is a validation process in which each individual case is classified using a function obtained from the total sample, excluding the individual case to be classified (Tabachnick & Fidell 1996). We made a correlation matrix with all morphometric variables to test for covariation between variables. If Pearson correlation value (R) was higher than 0.5, we selected arbitrarily the variable that resulted in the greatest separation in the *a posteriori* analysis. The amount of black in the cap and the ratio of bill length-width were not included in the analysis, given that both of them showed a correlation with the size of the rufous cap and the ratio of bill width-depth, respectively (CP*CB, $R^2 = -0.85$, $P < 0.0001$; BWD*BLW, $R^2 = -0.54$, $P < 0.001$).

RESULTS

Molecular sexing. PCR amplifications conducted using the P2 and P8 sexing primers to determine gender in individuals of Bay-capped Wren-Spinetails showed the typical pattern of bands that allowed us to clearly differentiate males from females. These primers amplify homologous sections of both conserved CHD (chromo-helicase-DNA-binding; CHD-Z and CHD-W) genes and incorporate introns of different lengths. When the fragment sizes were analyzed using a 3100 ABI automated sequencer, we detected a single CHD-Z peak 351 base-pairs long in males but females had a second larger and distinctive CHD-W peak 381 base-pairs long. We determined that this technique was 100% reliable for sex determination in this species because all 13 nest sites where breeding pairs of Bay-capped Wren-Spinetails were captured had, as predicted, one adult male and one adult female per nest.

Morphological differences between adult males and females. Males had a longer rufous cap (Fig. 1A) and wing chord than females, and the bill length-depth ratio values were higher in females than males (Fig. 1B), indicating that males had larger bill robustness than females (Table 1). The stepwise DFA selected four of the seven variables in the model: CR, WC, TL and BLD (Wilks' Lambda: 0.52, $F_{(4,29)} = 6{,}56$ $P < 0.0007$; Table 2). By using simultaneously these four morphometric variables, we obtained the following unstandardized discriminant function (D):

$$D = 0.40\,(CR) + 0.33\,(WC) + 0.88(TL) - 4.94\,(BLD) - 24.19$$

Individuals with discriminant function scores greater than the Eigenvalue (0.9) were classified as male and those with lower scores as female. Based on *a posteriori* analyses, this function correctly determined the sex of

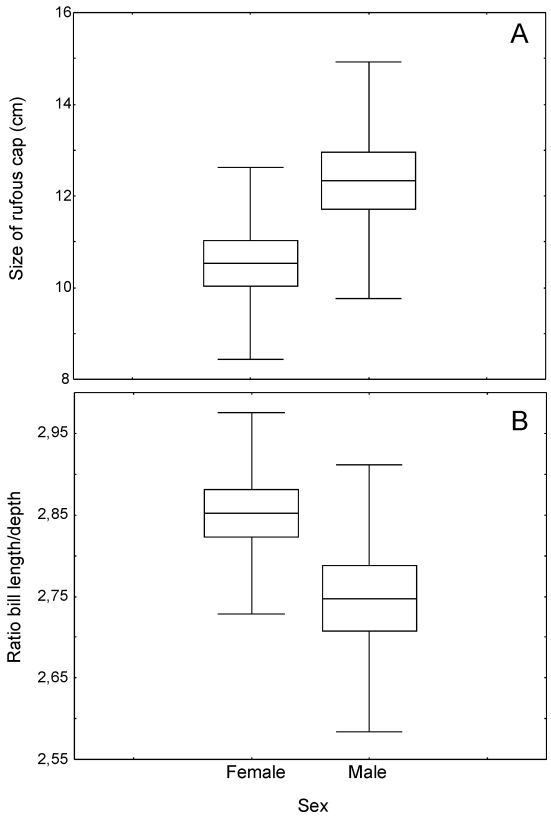

FIG. 1. Size of rufous cap (A) and ratio of bill length/depth (B) for females and males of *Spartonoica maluroides* inhabiting coastal saltmarshes from Argentina. Box plots are constructed with the limits of boxes being the 75th and 25th percentiles, and lines representing the 10th and 90th percentiles; lines inside the boxes are means.

80% of the overall samples, with an identification of 71 and 89% of males and females, respectively. The Jackknifed classification process provided had a similar sex classifications than those produced by discriminant analyses. The Jackknifed validation correctly determined the sex of 77% of the overall samples, with an identification of 71 and 82% of males and females, respectively.

DISCUSSION

We found a subtle sexual dimorphism in the Bay-capped Wren-Spinetail, because males average 15% larger in the length of the rufous cap and 2% larger in the wing chord than females. Moreover, the ratio of bill length-depth in males averaged 7% lower than females (i.e., males have a slightly more robust bill). The multivariate DFA distinguished females from males more efficiently than univariate comparisons of each character separately (Table 2). Because of the close morphological similarity of sexes of this species, the percentage of correct sex determination using single characters was always lower than when the discriminant function containing the four variables was used (CP, BLD, TL, and

TABLE 1. Morphometric measurements (mean ± SD) of Bay-capped Wren-Spinetails. *represent significant differences of t-test ($P < 0.05$)

Character	Females		Males		t-test	
	Mean (SD)	Range	Mean (SD)	Range	t-value	*P*
Ratio bill length/depth (BLD)	2.9 (0.1)	2.6–3.1	2.7 (0.2)	2.5–3	2.1	0.040*
Ratio bill width/depth (BWD)	0.8 (0.1)	0.6–1.2	0.8 (0.1)	0.7–0.9	0.4	0.659
Size cap rufous (CR; mm)	10.5 (2.1)	5.4–13.2	12.3 (2.6)	6.7–16.4	-2.3	0.029*
Tarsus length (TL; mm)	19.4 (0.4)	18.5–20.4	19.6 (0.4)	18.9–20.4	-1.6	0.116
Wing chord (WC; mm)	48.9 (1.5)	47–52	49.8 (1.0)	48–51	-2.0	0.049*
Tail length (TaL; mm)	63.8 (6.9)	50–73	63.1 (5.3)	54–73	0.3	0.761
Weight (W; g)	11.8 (0.8)	10.5–14	12.0 (0.6)	11–13.5	-0.6	0.560

WC; Table 2). Furthermore, the lack of correlation between the size measurements and the variables included in the discriminant function analysis suggest that overall discrimination is not based primarily on a difference in overall size.

The hypothesis for sexual bill dimorphism dates back to Selander (1966), who argued that where intraspecific competition is high and interspecific competition low, selection might favour divergence in foraging and diet between the sexes. These conditions arguably characterize populations of salt marsh song birds (Grenier & Greenberg 2006). There are alternative explanations to Selander (1966) regarding sexual size dimorphism. The obvious one is related to social or sexual selection. If males have to defend territories, a stronger bill may help in skirmishes over territory or fights directly for access to potential mates. However, the slight morphological and plumage coloration differences between sexes are consistent with a species and a family where sex role differences are minimal and deviations from strict social monogamy are few (Remsen 2003). Breeding and feeding behavior of Bay-capped Wren-Spinetail are poorly known, and it is currently not possible to test alternative functional hypotheses that might explain the bill shape difference between the sexes.

Although distinct rusty cap patches are common place among the various "Spinetail" taxa, until this study, none had been shown to be sexually dimorphic. Bright crown patches can vary among related taxa in whether their presence or size is related to gender. For example, the rusty crown patch is apparently similar between the sexes in various species of emberizid sparrows in the genus *Spizella*. However, the rusty crown patch is more frequently present and larger in males of the Swamp Sparrow (*Melospiza georgiana*; Greenberg 1988, Olsen & Greenberg in prep.). Interestingly, as in the case of *Spartonoica*, the Swamp Sparrow is one of the few marsh specialist species in its family, suggesting that future research might focus on the role of marsh habitat in selecting for a sexually dimorphic plumage badge in a convergent mode.

This study supports the proposition that the males of some ovenbirds are larger than females in morphometric characters (Winker *et al.* 1996, Remsen 2003, Roper & Hutson 2003, Faria *et al.* 2007, Moreno *et al.* 2007). Also, in order to detect of subtle and cryptic sexual dimorphism in putatively monomorphic species, future studies should pay closer attention to other characters that are not commonly used in morphometric studies such as iris color, organ size and muscle mass

TABLE 2. Value of correct determination of sexes in the Bay-capped Wren-Spinetail obtained by discriminant analysis using the four measurements separate and when they are included into the model and discriminant function.

Character	Wilks' Lambda	*F*	Female (%)	Male (%)	Total (%)
Size cap rufous (CR)	0.86	5.21	61	76	69
Ratio bill length/depth (BLD)	0.88	4.56	72	53	63
Tarsus length (TL)	0.93	2.6	78	53	66
Wing chord (WC)	0.88	4.19	61	71	66
Discriminant function (D)	0.52	4.29	89	71	80

(Murphy 2007), plumage UV reflectance (Tubaro *et al.* 2005; Eaton 2005, 2007) and the distribution and amount of rufous pigment in outer rectrices. Moreover, the results of this research are important to further analysis of geographic variation in morphology because apparent differences among sites may, without controlling for sex, be an artifact of local variation in sex sampling.

Finally, the ability to determine the sex of Bay-capped Wren-Spinetails using morphological and genetic methods will be useful for studies of mating systems and sexual selection, the habitat partitioning of sexes in the non-breeding season, heritability of morphological characters, differential dispersion and migration patterns, sexual differences in foraging behavior, vocalizations (Roper 2005) and in assessing effective population size in the often small and isolated populations of this threatened species.

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