Species and sex differences in vocalizations between sex-role reversed shorebirds, Northern Jacana *(Jacana spinosa)* and Wattled Jacana *(J. jacana)*

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ABSTRACT—Species-specific vocalizations can act as a reproductive isolating mechanism between closely related populations. We analyzed vocal differences between two hybridizing species of sex-role reversed polyandrous shorebirds, the Northern Jacana (*Jacana spinosa)* and Wattled Jacana (*J. jacana*). We found that Northern Jacana calls have higher fundamental frequency and peak frequency than Wattled Jacana calls. We also compared calls between females and males, as both jacana species are sex-role reversed and females compete for male mates. Males produce calls with a higher fundamental and peak frequency and shorter notes than females. These results suggest that vocal differences between Northern and Wattled Jacanas have the potential to act as a behavioral mediator of interspecific interactions, and that sex differences in vocalizations may relate to sex-role reversal in territorial defense and mate attraction.

Key words: hybridization, jacanas, sex-role reversal, sex differences, shorebird, vocalization

Diferencias específica y sexual en las vocalizaciones de las aves costeras de roles sexuales invertidos Jacana Norteña (*Jacana spinosa*) y Jacana Carunculada (*J. jacana*)

RESUMEN – Las vocalizaciones especie-específicas pueden actuar como mecanismos de aislamiento reproductivo entre poblaciones de especies estrechamente relacionadas. Analizamos las diferencias en vocalizaciones entre dos especies de aves costeras poliándricas de rol sexual invertido, *Jacana spinosa* y *Jacana jacana*. Encontramos que los llamados de *J. spinosa* contienen frecuencias fundamental y pico más altas que los llamados de *J. jacana*. También comparamos los llamados entre machos y hembras en ambas especies, ya que ambas tiene el rol sexual invertido y las hembras compiten por parejas. Los machos producen llamados con una frecuencia pico mayor y exhiben longitudes menores de notas que las hembras. Estos resultados sugieren que las diferencias en vocalizaciones podría actuar como barrera comportamental para limitar la hibridación entre las especies y estas vocalizaciones pueden funcionar distintamente entre machos y hembras de jacanas. Estudios futuros utilizando experimentos de reproducción de audio podrían poner a prueba estas hipótesis.

Palabras clave: ave costera, diferencia vocal, diferencias de sexo, hibridación, jacanas, llamada

Acoustic signals used to attract mates and repel competitors within a population may also influence mating outcomes between taxa (Slabbekoorn and Smith 2002, Price 2008, Uy et al. 2018). Various evolutionary processes can drive divergence in vocalizations among populations, including sensory drive (Derryberry 2009, Tobias et al. 2010), sexual selection (Hudson and Price 2014), cultural drift (Lachlan and Servedio 2004), reinforcement against maladaptive hybridization (Pfennig 2016), or a combination of these mechanisms (Wilkins et al. 2013). Alternatively, heterospecific vocalizations may converge due to shared habitat (Cardoso and Price 2010), song learning (Haavie et al. 2004), and/or selection for competitor recognition, i.e. agonistic character displacement (Grether et al. 2013, 2017), which can facilitate coexistence between taxa (Tobias et al. 2014, Kirschel et al. 2019). Hybrid zones – regions where distinct lineages come into contact and interbreed – provide a natural experiment (Hewitt 1988) to examine the causes and consequences of vocal differences for behavioral isolation (den Hartog et al. 2007, Lipshutz et al. 2017, Wheatcroft and Qvarnström 2017). Characterizing differences in vocalizations is an important first step in determining whether mating signals could serve to reproductively isolate lineages with otherwise incomplete barriers to gene flow.

Differences in spectral and temporal characteristics of vocalizations may also persist between the sexes. Larger body size and syrinx size is often associated with lower sound frequencies between the sexes and across species (Ryan and Brenowitz 1985, Ballintijn and ten Cate 1997, Barbraud et al. 2000). In most birds males are larger than females, but in species with female-biased size dimorphism, female vocalizations are lower pitched than male vocalizations (Taoka et al. 1989, Goymann et al. 2004, Maurer et al. 2008). Some taxa are exceptions to this rule; for example, many female owls are larger than males but have higher frequency calls (Herting et al. 2001, Odom and Mennill 2010). Within a species, low frequency vocalizations

may honestly signal body size and function in mate choice and/or intrasexual competition (Gil and Gahr 2002, Cardoso 2012), though this is not always the case (Cardoso et al. 2008). Sex-specific vocal behaviors may also relate to different social contexts, including courtship and territorial defense (Appleby et al. 2008, Catchpole and Slater 2008). For example, the sex that competes more for mates tends to vocalize more often (Sordahl 1979, Sung et al. 2005).

Females of some species are sex-role reversed, meaning they face stronger competition for mates than males do (Ah-King and Ahnesjö 2013). Sex-role reversal is also associated with female-biased size dimorphism (Emlen and Oring 1977), and larger body size can predict breeding success (Emlen and Wrege 2004). In sex-role reversed species, female vocalizations may indicate competitive ability and function in intrasexual competition. A study of sex-role reversed Black Coucals (*Centropus grillii*) found that females have higher call rates than males (Goymann et al. 2004). When challenged by playback simulating a territorial intrusion, female coucals sang with lower frequency and longer elements (Geberzahn et al. 2009), and these songs were perceived as more threatening (Geberzahn et al. 2010). In a study of another sex-role reversed species, the Bronze-winged Jacana (*Metopidius indicus*), males that called more often received more copulations (Butchart et al. 1999), suggesting that male vocalizations are also sexually selected. Currently, we know little about how the temporal and spectral characteristics of female and male vocalizations compare in sex-role reversed species.

Jacanas are tropical, sex-role reversed shorebirds in which selection on females to compete for mates is stronger than on males (Jenni 1974). The Northern Jacana (*Jacana spinosa*) and Wattled Jacana (*J. jacana*) have been isolated for around 700,000 yr (Miller et al. 2014) and hybridize in a narrow region in Panama (Lipshutz et al. 2019). The extent to which vocalizations differ between the species has not yet been quantified, and characterizing these differences can

help address questions about the role mating signals play in maintaining reproductive isolation between the species. Here, we quantified variation in temporal and spectral characteristics of vocalizations between the species and the sexes. We predicted that vocalizations between Northern and Wattled jacanas differ, and that the larger-bodied Northern Jacana would have lower frequency-related characteristics. Second, we examined vocal differences between males and females of both species. The sexes differ substantially in body size in jacanas, with body mass being up to 60% greater in females than males (Jenni and Collier 1972, Emlen and Wrege 2004). Because female jacanas are larger than males, we predicted that female vocalizations would have lower frequency-related characteristics, which could relate to sexual selection.

Methods

Sound recordings

We recorded vocalizations from June-August 2015 and June-July 2018 at 9 different sites in Panama (Fig. 1). Across these sites we recorded a total of 12 individuals of each species and sex, obtaining as many recordings per individual as possible. Birds were either stimulated with playback and a taxidermy mount to elicit vocalizations (Supplemental Fig. 1), or in some cases vocalizations were stimulated by the presence of the recordist near the bird's territory.

Recordings were made using a Marantz PMD661 MKII solid state digital recorder (Marantz professional, Cumberland, Rhode Island, United States) set at 44.1 kHz sampling rate, 16-bit, and WAV file type, and a Sennheiser K6 power module with a Sennheiser M67 shotgun microphone and windscreen (Sennheiser Electronic Corporation, Wedemark, Germany).

Acoustic measurement

Jacana vocalizations contain harmonics covering a wide frequency bandwidth (Jenni et al. 1974, Mace 1981, Jenni and Mace 1999). We took measurements on one call type, Repeated-note Call (RNC hereafter, Mace 1981), as these were consistently found in recordings of both species and sexes (Figure 2). RNCs are defined as a series of evenly spaced repeated sound elements (notes hereafter) less than 1 second apart. We divided continuous recordings for each individual into discrete RNCs in Audacity 2.1.2 (Audacity 2018). We used the sound-analysis software Luscinia (Lachlan 2007) to generate spectrograms (Figure 2). We high-pass filtered RNCs at 200 Hz to eliminate low frequency background noise. We used the following settings to measure RNC variation (abbreviations from Luscinia): Fundamental Frequency (FF) jump suppression = 20, Max. Frequency (Hz) = 15,000, Frame length (ms) = 5, Time step (ms) = 1, Spectrograph points = 221, Spectrogram Overlap % = 80, Dynamic range (dB) = 50, Dynamic equalization (ms) = 0, Dynamic comp. % = 100, Dereverberation % = 200, Dereverberation range (ms) = 100, Windowing function = Gaussian, Frequency zoom % = 150, Time zoom % = varies, Noise removal (NR) (dB) = 0, NR range1 (ms) = 50, NR range2 (ms) = 50.

We semi-automatically measured vocalizations in Luscinia (Lachlan 2007) by individually tracing each note within all complete RNCs obtained from each individual jacana (Supplemental Figure 2). Notes were traced by a single observer (EJB). We used Luscinia to extract 4 acoustic variables for each note: fundamental frequency (common denominator frequency of a harmonic signal, kHz), peak frequency (frequency of the maximum amplitude, kHz), note length (msec), and inter-note interval (msec). We also calculated two derived variables, note repetition rate (notes/sec) and duty cycle (% time emitting sound within an RNC). Note repetition rate was calculated by dividing number of notes within an RNC by the total length of each RNC (start of first note to end of final note) whereas duty cycle was calculated by

dividing the sum of note length for each RNC by total length (Clarkson 2007). We averaged these 6 variables across notes for each RNC per individual, except peak frequency, for which the median value is more representative of the actual peak frequency than the mean value (R. Lachlan, personal communication). We then calculated mean ± standard error of the 6 variables for each species/sex (Table 1) using R (R-Core-Team 2019).

Statistical analyses

We examined each of the 6 acoustic variables using linear mixed models with species and sex as fixed effects and site as a random effect using the lme4 package (Bates et al. 2015) in R. We initially included the interaction between species and sex as an additional fixed effect. For each acoustic dependent variable, we compared models with and without the interaction term using AIC_c and found that the model without the interaction term had a lower AIC_c. The interaction of species and sex was not a significant predictor of any acoustic variable. Therefore, we excluded the interaction term from all models. We $\log_{10}$ transformed frequency variables to approach the scale on which animals perceive and modulate sound frequency (Cardoso 2013). We also corrected for multiple comparisons using the Benjamini-Hochberg method with the p.adjust function in the R package 'stats' (R-Core-Team 2019).

We summarize the 6 acoustic variables for both species and sexes in boxplots representing minimum, first quartile, median, third quartile, and maximum in Figure 3, and in mean ± standard error in Table 1. We summarize fixed effect estimates (β), standard errors, Satterthwaite's method for estimating degrees of freedom, t-statistics, and p-values in Table 2. We also ran a discriminant function analysis (DFA) using JMP v15.1 (SAS Institute, Cary, NC) to assess if acoustic variables could distinguish the species and sexes.

Results

Description of note structure

The notes that comprise Northern Jacana RNCs are brief, broadband sound bursts with some harmonic structure. The most prominent feature of a note is typically a higher-energy portion (Fig. 2). Wattled Jacana RNCs are also composed of broadband sound bursts, but they contain more prominent harmonics than Northern Jacana notes. Within a note, these harmonics are often similar in amplitude, although the most energy is typically contained in the first or second lowest-frequency formant.

Species differences in vocalizations

RNCs of the two jacana species differ most in spectral characteristics. RNCs of Northern Jacanas are of significantly higher fundamental frequency and peak frequency than Wattled Jacanas, for both females and males (Table 1, Table 2, Fig. 3). The species did not differ in temporal characteristics of their RNCs, including note length, inter-note length, duty cycle, and note repetition rate. A DFA correctly classified all but 3 out of 48 individuals as the correct species (6.25% misclassification) based on all 6 acoustic variables. A forward, step-wise DFA identified fundamental frequency as the best variable to distinguish between the species (F ratio = 53.7, $p < 0.0001$).

Sex differences in vocalizations

RNCs of females and males differ in both temporal and spectral characteristics. For both species, RNCs of males are of significantly higher fundamental frequency and peak frequency than female RNCs (Table 1, Table 2, Fig. 3). RNCs of females contain longer notes and higher duty cycles. The sexes did not differ in inter-note length nor note repetition rate. A DFA correctly classified 35 out of 48 individuals as the correct sex (27.1% misclassification) based on all 6 acoustic variables. A forward, step-wise DFA identified note length as the best variable to distinguish between the sexes (F ratio = 9.8, p = 0.003).

Discussion

The Repeated-note Calls (RNCs) of Northern and Wattled jacana differ in the spectral variables we examined, fundamental and peak frequency, but not the temporal variables we examined. Female and male RNCs are consistently different across both species. RNCs of males are of higher fundamental and peak frequency, whereas notes are longer and duty cycles are higher in females.

Species differences

Counter to our predictions, the RNCs of the larger Northern Jacanas are of significantly higher fundamental and peak frequency than the RNCs of the smaller Wattled Jacanas. Body mass negatively correlates with acoustic frequency in many avian species examined (Ryan and Brenowitz 1985, Seddon 2005). However, differences in body mass between the two jacana species do not reflect differences in RNC frequency. A similar mismatch was found in *Corvus* crows, for which larger species have calls with higher frequency (Laiolo and Rolando 2003). This mismatch could also be driven by sex-differences: female Northern Jacanas have larger

202 body mass than female Wattled Jacanas, but males of these two species do not differ
203 significantly in body mass (Lipshutz 2017). Bill and syringeal morphology may instead explain
204 species differences in fundamental and peak frequency (Seneviratne et al. 2012, Kingsley et al.
205 2018), and future studies could compare these traits. Species differences in frequency-related
206 traits could also relate to differing environmental or habitat characteristics that have shaped their
207 call frequencies, in accordance with the acoustic adaptation hypothesis (Morton 1975, Endler
208 1992, Boncoraglio and Saino 2007).

209 In a prior study of the two jacana species, species distribution modeling indicated that
210 they have different habitat suitability (Miller et al. 2014). Future work could evaluate whether
211 the higher-frequency vocalizations of Northern Jacanas relate to a more open habitat. In contrast,
212 we did not find differences between the two species in the temporal variables we examined. A
213 similar pattern was found in a comparative study of auklets, in which frequency-related traits
214 differed more than temporal traits among species (Seneviratne et al. 2012). Altogether, finding
215 differences between the calls of these two hybridizing species suggests that future studies could
216 examine whether specific acoustic traits, such as peak frequency, could play a role in mediating
217 interspecific behavioral interactions.

218 In jacanas and other members of Charadriiformes, vocalizations are innate rather than
219 learned. Innate vocalizations may be particularly weak behavioral barriers to gene flow in
220 shorebirds, for which vocal repertoires and acoustic structure are highly conserved between sister
221 species and even among more distantly related groups (Miller and Baker 2009). There is mixed
222 evidence for the role of innate vocalizations as behavioral barriers to gene flow across species.
223 For example, innate vocalizations are functional barriers to hybridization in *Alectoris* partridges
224 (Ceugniet and Aubin 2001) and *Streptopelia* doves (de Kort et al. 2002), but not in *Callipepla*

225 (Gee 2005), nor *Coturnix* quails (Derégnaucourt and Guyomarc'h 2003). Our study does
226 demonstrate differences between the calls of these two hybridizing species, and a key empirical
227 question is whether males and females respond to these call differences in mating and
228 competitive contexts.
229
230 **Sex differences**
231 We found that sex differences in the fundamental and peak frequency of RNCs aligned with sex
232 differences in body mass; males of both species produce RNCs of higher frequency than the
233 larger-bodied females. In several species with female-biased size dimorphism, females have
234 lower-frequency calls than males (Goymann et al. 2004, Maurer et al. 2008). Lower frequency
235 RNCs in sex-role reversed jacanas could advertise female body size to male mates, which may
236 relate to fecundity selection (Pincheira-Donoso and Hunt 2017) and/or territorial defense (Emlen
237 and Wrege 2004). An open question is whether the lower-frequency calls of female jacanas are
238 merely a byproduct of sexual selection for increased body size due to their polyandrous mating
239 system, or whether there is evidence of direct sexual selection on this trait. Our study compares
240 the RNCs of not only males, but also females, adding to a growing body of research on female
241 vocalizations (Odom and Benedict 2018, Riebel et al. 2019).
242
243 **Application and conclusion**
244 We found that RNCs of Neotropical jacanas differ significantly in fundamental and peak
245 frequencies. Differences in vocalizations between the species could promote reproductive
246 isolation in their hybrid zone. These spectral characteristics also differed between the sexes,
247 suggesting that both male and female signals could facilitate species-specific discrimination in

248 the hybrid zone. Long-term goals in the Northern x Wattled jacana hybrid zone are to evaluate
249 the role of vocalizations in reproductive isolation. A previous phenotypic and genomic analysis
250 of the jacana hybrid zone found that species-specific traits such as plumage and facial
251 ornamentation are likely prezygotic barriers that maintain species boundaries (Lipshutz et al.
252 2019). This phenotypic differentiation between Northern and Wattled jacanas likely contributes
253 to the low occurrence of hybrids within the narrow hybrid zone and may be one of the reasons
254 for limited hybridization between the species. We were unable to evaluate whether a process
255 such as character displacement is influencing divergence in frequency traits, or convergence in
256 temporal traits, as we currently lack sufficient geographical sampling to compare vocalizations in
257 sympatry and allopatry. Future playback studies could assess the role of visual signals as
258 behavioral barriers to mating between the species by testing whether female and male jacanas are
259 more responsive to conspecific than heterospecific vocalizations.
260

261 ## Acknowledgments

262 This material is based on work supported by the University of Tennessee's Ready for the World
263 Chancellor's Honors Program and Summer Undergraduate Research Internship to EJB, as well
264 as the National Science Foundation under Grant No. 1154145 and IOS-1818235, and a
265 Smithsonian Tropical Research Institute short-term fellowship to SEL, Tulane University's
266 CELT Summer Research and Faculty-student Scholarly Engagement grants to TB, Tulane
267 University's Newcomb Scholars and Dean's summer research grants to GZ, and a Louisiana
268 Board of Regents NSF EPSCoR LINK Grant No. 177 to EPD. Any opinion, findings, and
269 conclusions or recommendations expressed in this material are those of the authors(s) and do not
270 necessarily reflect the views of the National Science Foundation. Scientific recording in Panama

was done with the approval of MiAmbiente (formerly ANAM), Panama's environmental authority (permit numbers: SE/A-45-12, SE/A-46-14, SE/A-17-18), and recording techniques were approved by the Institutional Animal Care and Use Committee of the Smithsonian Tropical Research Institute (STRI IACUC permits: 2012-0315-2015, 2018-0116-2021), Tulane University (IACUC permit: 0446R), and the University of Tennessee (IACUC permit: 2573). We thank the STRI Bird Collection for preparing taxidermy mounts. D.E. Buitrago and M. Araya Salas assisted with translating the abstract into Spanish. We thank S. McClelland for the jacana illustrations.

Data accessibility

Recordings are available on xeno canto: https://www.xeno-canto.org/set/4909

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Figure 1. Sampling map of Northern Jacana (yellow) and Wattled Jacana (red) vocalizations recorded across the hybrid zone Panama. Circle size represents sample size (minimum 1, maximum 19).

Figure 2. Spectrograms of male and female Northern and Wattled jacana vocalizations, displayed on an 8-kHz frequency scale. Each spectrogram represents a different individual.

Figure 3. Boxplots for acoustic variables in female (black) and male (grey) Northern Jacanas and Wattled Jacanas. Boxplots depict minimum, first quartile, median, third quartile, and maximum.

Table 1. Sampling information and mean ± standard error of spectral and temporal variables for Repeated-note Calls (RNCs) recorded from female and male Northern and Wattled jacanas.

Table 2. Linear mixed models testing differences in acoustic variables between species and sexes. Estimates (β) for species use Northern Jacanas as the reference and β for sex use males as the reference. Frequency variables are $\log_{10}$ transformed. P values adjusted (p_{adj}) with a Benjamini-Hochberg correction that are significant ($P<0.05$) are in bold.