

1 **Species and sex divergence in vocalizations between hybridizing role-reversed**
2 **shorebirds, Northern Jacana (*Jacana spinosa*) and Wattled Jacana (*Jacana***
3 ***jacana*)**

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12 **ABSTRACT**—Species-specific vocalizations can act as a reproductive isolating mechanism
13 between closely related populations. We analyzed vocal divergence between two hybridizing
14 species of sex-role reversed polyandrous shorebirds, the Northern Jacana (*Jacana*
15 *spinosa*) and Wattled Jacana (*Jacana jacana*). We found that *J. spinosa* calls have higher peak
16 frequency and fundamental frequency than *J. jacana* calls. We also compared calls
17 between males and females, as both jacana species are sex-role reversed and females compete for
18 male mates. Males produce calls with a higher peak frequency, exhibit shorter note lengths and
19 emit a greater number of notes within a calling bout than females, which could relate to mate
20 attraction. These results suggest that vocal divergence could act as a behavioral barrier to limit
21 hybridization between the species and vocalizations may function differently between male and
22 female jacanas.

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24 Key words: call, hybridization, jacanas, sex differences, shorebird, vocal divergence

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26 **Resumen. Divergencia específica y sexual en las vocalizaciones de las aves costeras de roles**
27 **sexuales invertidos Jacana Norteña (*Jacana spinosa*) y Jacana Carunculada (*Jacana***
28 ***jacana*).**

29 Las vocalizaciones especie-específicas pueden actuar como mecanismos de aislamiento
30 reproductivo entre poblaciones de especies estrechamente relacionadas. Analizamos la
31 divergencia en vocalizaciones entre dos especies de aves costeras poliándricas de rol sexual
32 invertido, Jacana Norteña (*Jacana spinosa*) y Jacana Carunculada (*Jacana jacana*). Encontramos
33 que los llamados de *J. spinosa* contienen frecuencias pico y fundamental más altas que los
34 llamados de *J. jacana*. También comparamos los llamados entre machos y hembras en ambas
35 especies, ya que ambas tiene el rol sexual invertido y las hembras compiten por parejas. Los
36 machos producen llamados con una frecuencia pico mayor, exhiben longitudes menores de notas
37 y emiten un número mayor de notas dentro de un despliegue de vocalizaciones y producen notas
38 de menor duración que las hembras, lo que podría relacionarse con atracción de pareja. Estos
39 resultados sugieren que la divergencia en vocalizaciones podría actuar como barrera
40 comportamental para limitar la hibridación entre las especies y estas vocalizaciones pueden
41 funcionar distintamente entre machos y hembras de jacanas. Estudios futuros utilizando
42 experimentos de reproducción de audio podrían poner a prueba estas hipótesis.

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

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45 Between closely related species, divergence in mating signals can facilitate reproductive
46 isolation and drive the process of speciation (Irwin and Price 1999; Coyne and Orr 2004).
47 Whereas mating signals are used to attract and compete for mates within populations, divergence
48 between populations can lead to a breakdown in communication such that individuals do not
49 recognize potential mates or rivals (Coyne and Orr 2004). Evidence from a wide range of taxa
50 suggests that the degree of divergence in mating signals influences the extent to which
51 individuals discriminate between congeners, which ultimately shapes mating outcomes
52 (Andersson 1994). Therefore, it is important to understand how and why mating signals diverge
53 between populations. Hybrid zones – regions where distinct species come into contact and
54 interbreed – provide a natural experiment to examine the consequences of vocal divergence for
55 behavioral isolation (Hewitt 1988).

56 Divergent mating signals can serve to reproductively isolate species with otherwise
57 incomplete barriers to gene flow (Grant and Grant 1997; Price 2008). Learned vocalizations,
58 such as oscine songs (Nottebohm 1972), have the potential to diverge rapidly via cultural
59 evolution (Mason et al. 2016) and are therefore important pre-mating barriers to gene flow
60 between hybridizing populations (Slabbekoorn and Smith 2002; Uy et al. 2018). In contrast,
61 innate vocalizations diverge more slowly than learned vocalizations, and there is mixed evidence
62 for the role of innate vocalizations as behavioral barriers to gene flow. For example, innate
63 vocalizations serve as behavioral barriers to hybridization in *Alectoris* partridges (Ceugniet and
64 Aubin 2001) and *Streptopelia* doves (De Kort et al. 2002), but not in *Callipepla* (Gee 2005), nor
65 *Coturnix* quails (Derégnaucourt and Guyomarc'h 2003). This leaves an important gap in our
66 understanding about innately derived vocalizations and how they vary between closely related
67 species that are hybridizing.

68 In many bird species, both males and females vocalize (Odom and Benedict 2018). Vocal
69 traits such as note length, complexity or production rate may differ between the sexes depending
70 on their function in courtship and other behavioral contexts (Appleby et al. 1999; Odom and
71 Mennill 2010; ten Cate 1997). For example, the sex that competes more for mates tends to
72 vocalize more often (Sordahl 1979; Sung et al. 2005). Whereas male competition for mates is
73 common across animals, females of some species are sex-role reversed, meaning they face
74 stronger competition for mates than males do (Emlen and Oring 1977). Currently, we know very
75 little about how female and male vocalizations compare in sex-role reversed species.

76 Sexually dimorphic vocalizations may also occur due to physical differences between
77 males and females. In many species the sexes diverge in body size. As a consequence,
78 morphological constraints on sound production can lead to distinctive spectral and temporal
79 characteristics between male and female vocalizations (Ryan and Brenowitz 1985; Ten Cate
80 1997). Consistent with signal design theory, larger body size is often associated with lower
81 sound frequencies both between and within sexes (Barbraud et al. 2000; Maurer et al. 2008).
82 Some taxa are exceptions to this rule; for example, many female owls are larger than males but
83 have higher frequency calls (Odom and Mennill 2010). Therefore, it is not clear whether signal
84 design theory will hold in sex-role reversed species, in which females are often larger than
85 males.

86 Jacanas are tropical, sex-role reversed shorebirds in which selection on females to
87 compete for mates is stronger than on males (Jenni 1974). The Northern Jacana (*Jacana spinosa*)
88 and Wattled Jacana (*J. jacana*) have been isolated for 700,000 years (Miller et al. 2014) and
89 hybridize in a narrow region in Panama (Lipshutz et al. 2019). It is unknown whether their
90 vocalizations are divergent, and what role their calls play in maintaining reproductive isolation

91 between the species. Here, we quantify variation in temporal and spectral characteristics
92 between the species and the sexes. We predict that vocalizations between *J. spinosa* and *J.*
93 *jacana* will be divergent, and that the larger-bodied *J. spinosa* will have lower frequency-related
94 characteristics. Second, we examine vocal divergence between males and females of both
95 species. Jacanas have extreme sexual dimorphism in size, and females weigh up to 60% more
96 than males (Emlen and Wrege 2004; Jenni and Collier 1972). Because female jacanas are larger
97 than males, we predict that female vocalizations will have lower frequency-related
98 characteristics. We also predicted that females should produce more calls than males, given that
99 these species are sex-role reversed.

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Methods

102 Sound recordings

103 We recorded vocalizations from June-August 2015 and June-July 2018 at 9 different sites in
104 Panama (Fig. 1). Across these sites we recorded a total of 12 individuals of each species and sex.
105 Birds were either stimulated with playback and a taxidermic mount to elicit vocalizations, or in
106 some cases vocalizations were stimulated by the presence of the recordist near the bird's
107 territory.

108 Recordings were made using a Marantz PMD661 MKII solid state digital recorder
109 (Marantz professional, Cumberland, Rhode Island, United States) set at 44.1 kHz sampling rate,
110 16-bit, and WAV file type, and a Sennheiser K6 power module with a Sennheiser M67 shotgun
111 microphone and windscreen (Sennheiser electronic corporation, Wedemark, Germany). We
112 divided continuous recordings for each individual into discrete call bouts (average = 6.1, range =

113 1-32 call bouts per individual) in Audacity 2.1.2 (Audacity 2018). Call bouts are defined as a
114 series of evenly spaced notes less than 1 second apart.

115 **Acoustic measurement**

116 Jacana vocalizations contain harmonics covering a wide frequency bandwidth (Mace 1981). We
117 took measurements on one call type, repeated note calls (Jenni 1974), as these were consistently
118 found in recordings of both species and sexes (Fig. 2). We used the sound-analysis software
119 Luscinia (Lachlan 2007) to generate Fourier-based spectrograms. Calls were high pass filtered to
120 eliminate low frequency background noise below 200 Hz. We used the following settings to
121 measure call variation: FF jump suppression = 20, Max. Frequency (Hz) = 15,000, Frame length
122 (ms) = 5, Time step (ms) = 1, Spectrograph points = 221, Spectrogram Overlap % = 80, Dynamic
123 range (dB) = 50, Dynamic equalization (ms) = 0, Dynamic comp. % = 100, Dereverberation % =
124 200, Dereverberation range (ms) = 100, Windowing function = Gaussian, Frequency zoom % =
125 150, Time zoom % = varies, Noise removal (dB) = 0, NR range1 (ms) = 50, R range2 (ms) = 50.

126 We manually measured vocalizations in Luscinia (Lachlan 2007) by individually tracing
127 each note. We used the Luscinia software to automatically calculate three acoustic parameters:
128 note length (msec), peak frequency (frequency of the maximum amplitude, Hz), and fundamental
129 frequency (Hz). We averaged these parameters for each note within a call bout (range 2-70 notes,
130 mean 11.6) (Table 1). We also averaged the number of notes per bout for each individual to
131 calculate a fourth acoustic parameter, notes per bout. The four parameters were averaged in
132 Microsoft Excel after exporting from Luscinia.

133 **Statistical analysis**

134 We compared the four call parameters between the species and sexes using Student's *t*-tests or
135 Wilcoxon rank sum, depending on whether the parameters were normally distributed or not.

136 We also summarized the four acoustic parameters with a principal components analysis
137 (PCA) using the `prcomp` function in R version 3.3.2 (R-Core-Team, 2015). Prior to the PCA, we
138 log-transformed acoustic data to fulfill assumptions of multi-normality. We retained two PC
139 scores that explained 60.6% and 19.7% of the variation in the acoustic parameters, totaling
140 80.7% cumulative proportion of variation (Table S1). Frequency variables loaded negatively
141 onto PC1 and positively onto PC2. Note length loaded positively on PC1 and PC2, and notes per
142 bout loaded negatively onto PC1 and PC2. We compared vocalizations between the species and
143 sexes using linear mixed effects models with the `nlme` package (Pinheiro et al. 2018) in R. We
144 included vocalization PC1 and PC2 as separate response variables, species and sex as the fixed
145 effects, and site as a random effect. We visually inspected residual plots to ensure they did not
146 deviate from normality. We used a type III ANOVA to determine whether species and/or sex
147 were significant predictors of variation in the PCs.

148 We also conducted a discriminant function analysis (DFA) in R using the package
149 `flipMultivariates` to assess whether spectral or temporal parameters could distinguish between
150 species and sexes (<https://github.com/Displayr/flipMultivariates/>).

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Results

Species differences in vocalizations

154 Vocalizations are different between these two jacana species, particularly regarding spectral
155 characteristics (Table 1; Fig. 3). For both males and females, *J. spinosa* calls have significantly
156 higher peak ($t = -5.1$, $df = 41.8$, $P < 0.001$) and fundamental frequencies ($W = 42$, $P < 0.001$)
157 than *J. jacana* calls. Species was also a significant predictor both of vocalization PC1 ($F_{1,7} =$
158 24.8 , $P = 0.002$) and PC2 ($F_{1,7} = 16.7$, $P = 0.005$) (Fig. S1). Using all acoustic parameters, 44 out

159 of 48 (92%) individuals were classified to the correct species by a DFA. The best variables to
160 distinguish the species were peak frequency ($r^2 = 0.36$, $P < 0.001$) and fundamental frequency (r^2
161 $= 0.48$, $P < 0.001$).

162 **Sex differences in vocalizations**

163 Vocalizations are also different between males and females of both species (Table 1; Fig. 3).
164 When species are combined, males have significantly more calls within a bout ($W = 189.5$, $P =$
165 0.043) and higher peak frequency calls ($t = -2.7$, $df = 44.9$, $P = 0.009$) than females. Females
166 have longer calls than males ($t = 3.1$, $df = 40.7$, $P = 0.003$). Sex was also a significant predictor
167 of vocalization PC1 ($F_{1,38} = 14.8$, $P = < 0.001$) but not of PC2 (Fig. S1). Using all acoustic
168 parameters, 45 out of 48 (73%) individuals were classified to the correct sex by a DFA. The best
169 variables to distinguish the sexes were call length ($r^2 = 0.17$, $P = 0.005$), peak frequency ($r^2 =$
170 0.14 , $P = 0.014$), and calls within a bout ($r^2 = 0.12$, $P = 0.028$).

171 The sexes were more strongly differentiated by peak frequency in *J. jacana* ($t = -3.0193$,
172 $df = 21.98$, $P = 0.006$) than in *J. spinosa* ($t = -1.9602$, $df = 21.999$, $P = 0.063$). Similarly, males
173 and females differed more strongly in note length in *J. jacana* ($t = 3.3939$, $df = 19.602$, $P =$
174 0.003) than in *J. spinosa* ($t = 1.6899$, $df = 19.318$, $P = 0.11$). In contrast, the sexes differed more
175 strongly in calls within a bout for *J. spinosa* ($W = 38.5$, $P = 0.057$) than *J. jacana* ($W = 53$, $P =$
176 0.29).

177

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Discussion

179 *Jacana spinosa* and *J. jacana* calls are different - *J. spinosa* calls are significantly higher
180 in peak and fundamental frequency. The sexes are consistently different across both species,

181 such that male calls have higher peak frequency and more notes than female calls, whereas
182 female calls have longer note lengths.

183 **Species Differences**

184 Vocalizations of the two species of jacana have diverged spectrally but are similar temporally. In
185 other species of non-oscine birds that do not learn their songs, temporal traits change at slower
186 rates than frequency-related traits (Miller and Baker 2009, Seneviratne et al. 2012), which is
187 consistent with our findings that spectral traits were more divergent than temporal traits between
188 these jacana species. Counter to the prediction that smaller-bodied species should have calls with
189 higher frequencies (Ryan and Brenowitz 1985), larger-bodied *J. spinosa* have higher peak and
190 fundamental frequency vocalizations than smaller-bodied *J. jacana*, for both sexes. One
191 hypothesis for this contradiction is that species divergence in vocalizations could relate to
192 differing environmental or habitat characteristics that have shaped their call frequencies (Morton
193 1975, Endler 1992). In a prior study, species distribution modeling indicated that *J. spinosa*
194 favors a warmer and wetter environment (Miller et al. 2014), suggesting that the species have
195 diverged in habitat preferences. Future work could compare the vegetative cover in the habitat of
196 each species to determine whether the higher frequency vocalizations of *J. spinosa* relate to a
197 more open habitat. Another potential explanation for vocal divergence could be a difference in
198 the syringeal or bill structure between species (Seneviratne et al. 2012; Kingsley et al. 2018).

199 **Sex Differences**

200 We found that frequency differences in jacana vocalizations matched our predictions for females
201 and males based on their body size dimorphism; males of both species produce higher frequency
202 calls than the larger-bodied females. In many species with sexually dimorphic body size, the
203 smaller of the two sexes produces higher frequency vocalizations (Maurer et al. 2008). Female-

204 biased size dimorphism is common in shorebirds other than jacanas, and larger females also have
205 lower frequency calls (Heidemann and Oring 1976; Douglas 1998). Contrary to our expectation,
206 male jacanas within our study also emitted more notes per calling bout than females. One likely
207 explanation for this is mate attraction: in the polyandrous Bronze-winged Jacana (*Metopidius*
208 *indicus*), individual males that called more frequently received more copulations than other co-
209 mates (Butchart et al. 1999). Calling rate could be a sexually selected trait in male Neotropical
210 jacanas, and this hypothesis should be tested using behavioral playback experiments.

211 **Application and conclusion**

212 We found that Neotropical jacanas diverged significantly in the peak and fundamental
213 frequencies of their vocalizations. Diverged vocal signals could promote reproductive isolation
214 between the two species when they come into contact. Furthermore, these spectral characteristics
215 differed between the species for both sexes, suggesting that both male and female signals could
216 facilitate species-specific discrimination in the hybrid zone. A phenotypic and genomic analysis
217 of the jacana hybrid zone found that species-specific traits such as plumage and facial
218 ornamentation were likely prezygotic barriers that maintain species boundaries (Lipshutz et al.
219 2019). Future playback studies could assess the relative role of visual and vocal signals as
220 behavioral barriers to mating between the species. This phenotypic differentiation between *J.*
221 *spinosa* and *J. jacana* likely contributes to the low occurrence of hybrids within the narrow
222 hybrid zone and may be one of the reasons for limited hybridization between the species.

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242

243 **Data accessibility**

244 Recordings are available on xeno canto. *Jacana spinosa*: <https://www.xeno-canto.org/set/4909>

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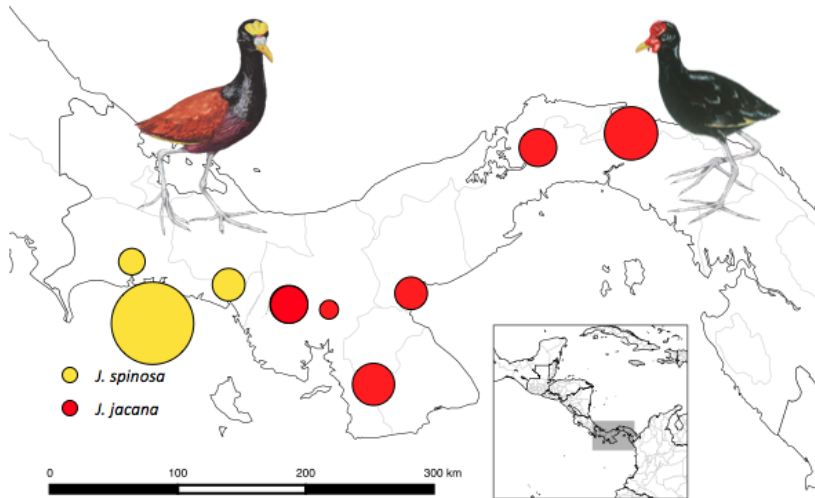
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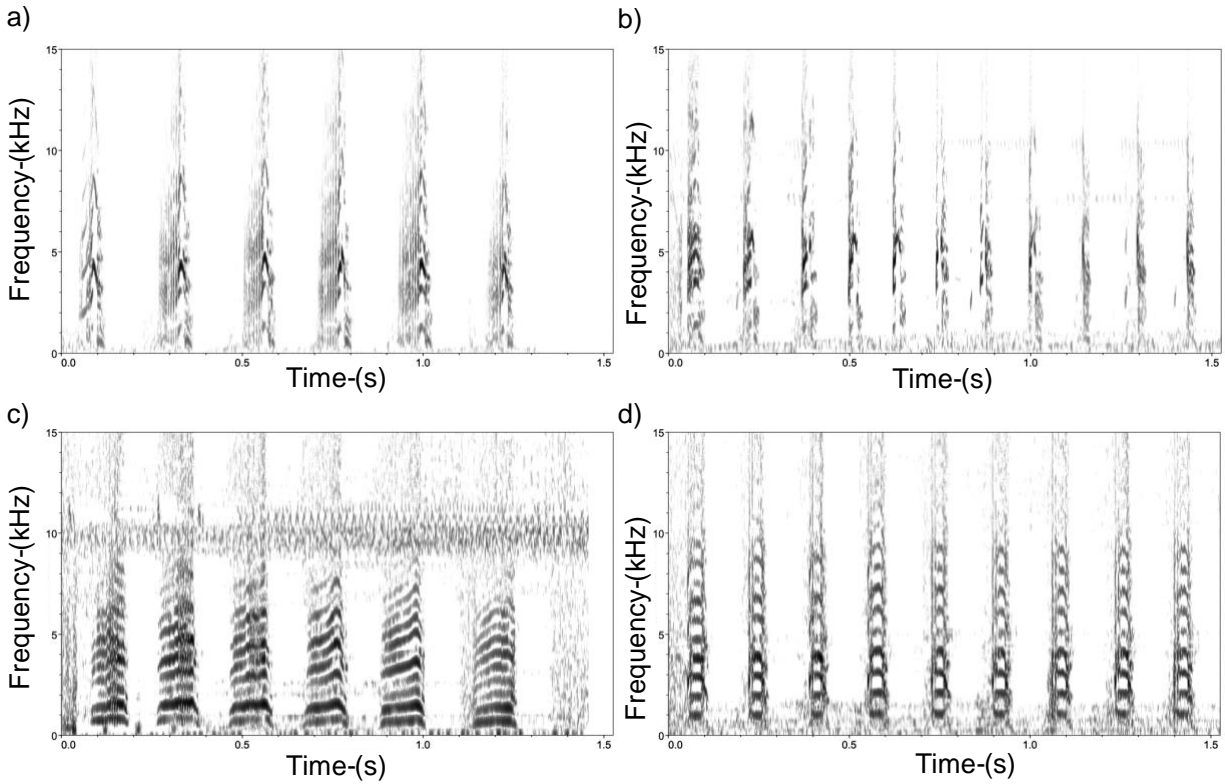
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349

350 **Figure 1.** Sampling map of *Jacana spinosa* (yellow) and *J. jacana* (red) vocalizations recorded

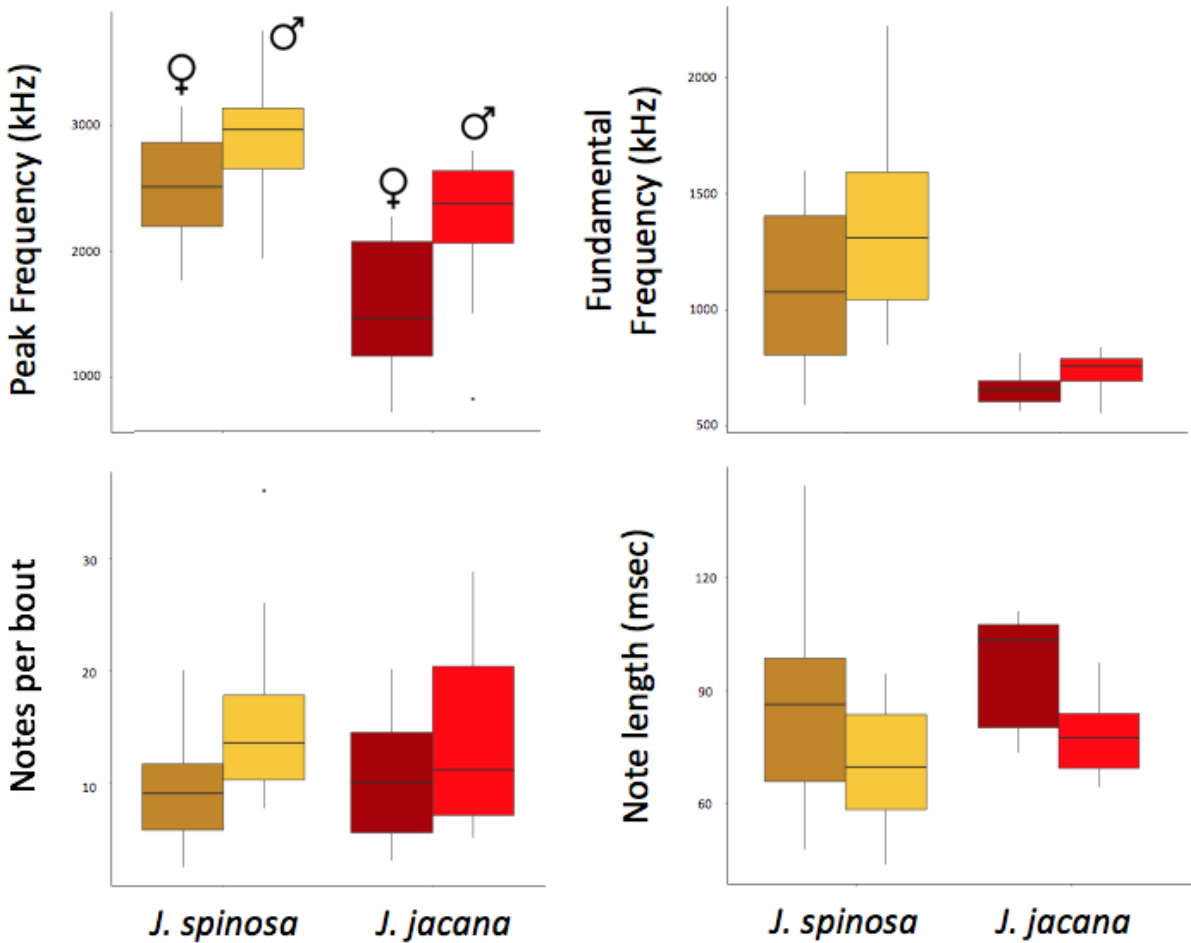
351 across the hybrid zone Panama. Circle size represents sample size (minimum 1, maximum 19).



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353 **Figure 2.** Spectrograms of vocalizations for a) female *J. spinosa*, b) male *J. spinosa*, c) female *J.*

354 *jacana*, d) male *J. jacana*



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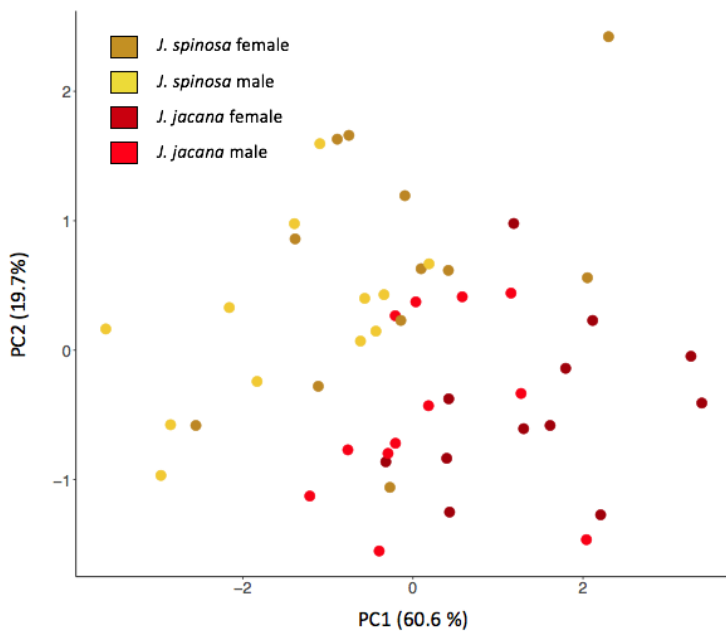
356 **Figure 3.** Mean spectral and temporal characteristics for vocalizations recorded from male
 357 (light) and female (dark) *Jacana spinosa* (yellow) and *J. jacana* (red).

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Table 1. Sampling information and spectral and temporal characteristics (mean \pm SE) for vocalizations recorded from male and female *Jacana spinosa* and *J. jacana*.

	<i>Jacana spinosa</i>		<i>Jacana jacana</i>	
	Male	Female	Male	Female
Number of Individuals	12	12	12	12
Notes per Bout	15.97 \pm 2.36	9.75 \pm 1.55	14.03 \pm 2.42	10.32 \pm 1.66
Range of Notes per Bout	2 – 45	2 – 33	3 – 70	2 – 68
Note Length (ms)	70.17 \pm 5.09	85.51 \pm 7.52	78.12 \pm 2.98	95.87 \pm 4.3
Peak Frequency (Hz)	2898.36 \pm 129.97	2539.14 \pm 129.2	2221.52 \pm 166.34	1521.63 \pm 161.45
Fundamental Frequency (Hz)	1346.54 \pm 112.46	1099.14 \pm 99.4	731.83 \pm 24.71	663.84 \pm 23.46

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361 **Supplemental Figure 1.** Principal components analysis of *Jacana spinosa* (yellow) and *Jacana*
362 *jacana* (red) males (light) and female (dark) vocalizations

Supplemental Table 1. Loadings for Principal Component Analysis

Vocal Parameter	PC1	PC2
Eigenvalue	1.56	0.89
Proportion of variance	60.60%	19.70%
Note per bout	-0.47	-0.58
Note length (ms)	0.49	0.45
Peak frequency (Hz)	-0.53	0.45
Fundamental frequency (Hz)	-0.52	0.5

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