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1 **Water restriction influences intra-pair vocal behavior and the acoustic structure of**
2 **vocalisations in the opportunistically breeding zebra finch (*Taeniopygia guttata*)**

3
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14 Running Head: Effect of water restriction on zebra finch vocal behavior

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23 **Introduction**

24 Temperate seasonally-breeding songbirds experience profound fluctuations in singing
25 associated with the changing seasons (Slagsvold 1977; Catchpole and Slater 2008). More
26 broadly, there is growing evidence from many species which suggests there are widespread
27 changes to auditory-vocal communication systems throughout the year: including variation in the
28 type and composition of songs and calls (Marler 1956; Brenowitz 2004; Marler 2004; Maddison
29 et al. 2012; Lohr et al. 2013), acoustic structure of song syllables (Maddison et al. 2012), and
30 auditory perception (Lucas et al. 2007; Bass et al. 2016; Caras and Ramage-Healey 2016).

31 Whereas, a higher amount of courtship song during breeding seasons can easily be described as
32 an example of breeding behavior coupled to the appropriate environmental context, the
33 functional significance of these other changes in auditory-vocal communication systems remain
34 largely unknown.

35 For opportunistically breeding species, it is unclear how environmental condition alone
36 induces any changes in auditory-vocal communication systems, such as is seen in seasonally-
37 breeding species. The Australian zebra finch is an opportunistically breeding species. Zebra
38 finches time breeding bouts based on many environmental factors such as food and water
39 availability, the presence of green grasses, humidity and day length (Perfito 2010). However,
40 zebra finches within the same population, under good environmental conditions, are not
41 necessarily all in the same breeding state (Perfito 2010). Furthermore, in the wild, zebra finch
42 pairs do not always breed synchronously (Zann 1996). In the laboratory, experimental water
43 restriction has reliably been used as a method for manipulating male and female breeding
44 readiness (Perfito et al. 2006; Prior et al. 2013; Prior and Soma 2015). The physiological effects
45 of water restriction on males and females are somewhat varied; however, both sexes do

46 experience similar shifts in neuroendocrine state as what has been described for seasonally
47 breeding species (e.g. brain and circulating steroid levels) (Perfito 2010; Prior et al. 2013; Perfito
48 et al. 2015; Prior and Soma 2015).

49 While zebra finches will engage in courtship behaviors throughout the year, the fact that
50 they coordinate breeding bouts at the level of the pair raises the question of whether shifts in
51 vocal dynamics, that proceed breeding, could be used to coordinate breeding attempts. Indeed
52 there is some evidence that vocal behavior may shift with environmental condition in zebra
53 finches, as well as evidence that there are season-like shifts in neuroendocrine state centrally,
54 which could mediate season-like behavioral variation (Prior and Soma 2015; Perfito et al., 2015).

55 For wild zebra finches, there is evidence that undirected song is more common during non-
56 breeding periods (Dunn and Zann 1996a; Dunn and Zann 1996b). Additionally, during periods of
57 active nesting zebra finches produce a synchronized vocal exchange (duet) that is used to
58 coordinate bi-parental care (Elie et al. 2010; Boucaud et al. 2016; Boucaud et al. 2017). More
59 broadly, there is a shift in call types used during nesting (Gill et al. 2015), and some call types
60 are used almost exclusively during nesting (Elie et al. 2010; Gill et al. 2015).

61 Here we tested the effect of water restriction, as an environmental manipulation that is
62 known to impact breeding, on three aspects of zebra finch vocal behavior: (1) overall vocal
63 activity, (2) patterns of vocal exchanges, and (3) the acoustic structure of vocalizations. We
64 manipulated breeding readiness using established experimental water restriction paradigms. In
65 order to compare the effect of water restriction to previous studies, we quantified circulating
66 testosterone in males and circulating corticosterone levels in males and females. Despite that
67 zebra finches are gregarious, they actually appear to spend the majority of their life with their
68 pair bonded mate (Zann 1996; McCowan et al. 2015); thus, we chose to focus on describing the

69 effects of water restriction on the vocal behavior of well-established zebra finch pairs.
70 Additionally, in order to control for social condition during recordings, we recorded vocal
71 behavior while pairs were visually isolated (Perez et al. 2015b). This forced all intra-pair
72 communication to be through the vocal-acoustic domain and not visual or tactile.

73

74 **1. Materials and Methods**

75 *2.1 Subjects*

76 Twenty-eight paired adult domesticated zebra finches (>120 days old) were used for this
77 study. All pairs were bonded for >1 year and had been allowed to breed together for several
78 breeding cycles. Thus, only successfully-bonded pairs were used. Each pair was housed in their
79 own cage (dimensions 50.8 × 40.5 × 30.5 cm). The colony was maintained on a 14:10 h light:
80 dark cycle with temperatures between ~24-26 °C. All individuals had *ad libitum* access to seed,
81 cuttlefish bone, and grit in addition to supplemental green vegetables once per week. Note that
82 over the course of the study one individual died, fifteen pairs were recorded in the control
83 condition (CON), while only fourteen of these pairs were also recorded in the water restricted
84 condition (WR).

85

86 *2.2 Experimental Design*

87 Water availability was manipulated in order to influence reproductive condition. Pairs
88 were slowly water restricted over the course of six weeks from 5mL down to 1mL per individual
89 per week. This protocol is similar to what has previously been published (Prior et al. 2013; Prior
90 et al. 2014). Briefly, at the start of water restriction, two empty water towers were placed on each
91 cage. Initially, 2mL of water was administered via pipette into each water tower five days a

92 week. The amount of water administered was gradually decreased to .5mL twice a week in each
93 water tower.

94 Vocal behavior of each pair was recorded three times during Water Restriction (WR) and
95 three times during Water *ad libitum* (CON) resulting in six recordings/pair (each recording ~4
96 hours). From these recordings, we quantified the overall vocal activity (song and call rate), the
97 quality of vocal exchanges (male song composition and patterns of intra-pair calling exchanges),
98 and the acoustic structure of vocalisations (Figure 1). To control for potential confounds of
99 habituation to the recording session, the order of water restriction was counterbalanced across
100 pairs: nine pairs were recorded first under the CON and then under the WR condition (Cohort 1),
101 and six pairs were recorded first under the WR and then under the CON condition (Cohort 2).
102 For both cohorts there was a break (37 days for cohort 1 and 16 days for cohort 2) between
103 conditions. Importantly, this amount of time (>2 weeks) has previously been shown to allow
104 recovery from water restriction (Vleck and Priedkalns 1985). In order to assess the effect of
105 water restriction on systemic hormone levels, we collected blood samples from the brachial vein
106 and quantified circulating corticosterone and testosterone levels from each individual 0-3 days
107 following the completion of recordings for each condition (CON and WR). Thus, there were at
108 least 2 weeks between blood sample collection and the acoustic recordings.

109

110 *2.3 Recording vocal behavior*

111 To identify effects of water restriction on acoustic communication, we recorded intra-pair
112 vocal behavior while the male and female of each pair were visually isolated. Since the only
113 means of contact between partners during visual separation is acoustic, this paradigm allowed us
114 to disentangle effects of our environmental manipulation on acoustic communication from more

115 general pairing behaviors. Previous research has shown that intra-pair vocal dynamics are more
116 stereotyped while visually isolated (Perez et al. 2015b). While visually-isolating pairs introduces
117 a perturbation that could itself impact behavior, this isolation removes confounds that could
118 result from effects of water restriction on other sensory systems involved in communication
119 dynamics.

120 Each pair was moved from their home cage to a sound attenuation chamber (cage
121 dimensions $63.5 \times 33.0 \times 30.5$ cm) the day before recordings (~14:00 h). The male and female
122 were physically separated in the testing chamber by placing each bird in individual but adjacent
123 cages. Each cage contained two perches, a seed cup and a water tower, which was either filled or
124 empty depending on water condition. Initially pairs remained in visual contact to facilitate
125 habituation to the testing chamber. After ~ 3 h, an opaque partition was placed between the two
126 cages (Figure 1). Behavior was not recorded until the following day which ensured behavior was
127 not recorded immediately after the stress of handling and moving. In total pairs had at least 15 h
128 to habituate to the testing set up.

129 The following morning, passive recordings of each pair were made using a digital
130 recorder (SongMeters SM2, 16-bit, 44 kHz sampling rate; Wildlife Acoustics Inc., Concord,
131 MA, U.S.A.) with omnidirectional microphones (SMX-II, Wildlife Acoustics Inc.;
132 omnidirectional, flat frequency response 20 Hz-20 kHz, sensitivity 36 ± 4 dB, 0 dB 1/4 1 V/pa at
133 1 kHz). One microphone was positioned above each partner's cage. More specifically, the
134 microphone was positioned downwards above the cages in order to minimize amplitude
135 variations due to the position of the bird. Each recording was ~4 h long from ~08:00-12:00, the
136 period starting at lights on. There were six recordings in total (Recording number (Nb), 1-6),
137 with ~12 h of recordings/pair/water condition.

138

139 *2.4 Scoring Vocal Behavior*

140 All vocalisations were extracted from recordings using in-house software, as previously
141 described (Elie et al. 2011; Perez et al. 2015b) (see supplemental information).

142

143

144 2.4.1 Songs

145 Songs were identified manually (see supplemental information). In total we recorded 205
146 songs from 13 males during CON and 28 songs from 8 males during WR. Characteristics of each
147 song were manually scored (N.H.P.) using Praat (V 5.3.56, 2014) (Boersma and Weenik 1996).
148 Song bouts were defined as a single rendition of song, separated by at least 1 sec. Because there
149 were drastically fewer WR songs and most males did not sing on each recording date we
150 analyzed song rate by calculating a single song rate per male for each water condition (CON and
151 WR).

152 In order to determine the effect of water restriction on the composition and acoustic
153 structure of song, we further processed song in two ways. First, global song elements were
154 extracted manually, as concise sequences, in Praat. More specifically, the following sequences
155 were extracted: an introductory note sequence as well as every full and partial motif (Figure 2A).
156 Any additional inserted elements (e.g. distance calls) were not extracted for analysis. Second, the
157 following four individual notes were extracted: the first and last introductory notes, as well as
158 two song syllables: one from the first half of the motif (early syllable) and the second from the
159 second half of the motif (late syllable) (Figure 2B). For a given male, the same two song

160 syllables were extracted from each song bout. We preferentially selected harmonic, ‘stacked’
161 syllables to facilitate consistent labeling of the note boundaries.

162 We used the extracted global song elements to generate four characteristics of song: (1)
163 mean duration of full motifs, (2) the ratio of the duration of full motifs to the duration of the total
164 song bouts (the duty cycle), (3) the mean inter-sequence interval (between the introductory note
165 sequence of first motif and subsequent motifs) and (4) the ratio of the duration of the
166 introductory note sequence to the total duration of motifs in the corresponding song bout.
167 Additionally, analyses of song composition were performed on the full and partial motifs (*see*
168 *supplemental information*). The extracted introductory notes and song syllables were used for
169 acoustic structure analysis (*see supplemental information*).

170

171 2.4.2 Calls

172 In addition to song, we extracted all the calls during a recording. Our initial observations
173 suggested that pairs engaged in call and response periodically throughout the recordings and that
174 during quieter periods there was typically only one individual calling. Thus, in order to focus on
175 intra-pair calling, we defined calling ‘bursts’, where calling activity was elevated (overall call
176 rate per pair was >10% above the average for the recording) (*see supplemental information*). By
177 isolating bursts specifically for analysis, we increased the likelihood that we were examining
178 parameters of true vocal exchanges. We quantified burst rate (# bursts/ hour) and call rate per
179 individual (for the full recording and within bursts specifically), as well as two parameters of
180 calling exchanges: (1) temporal coordination (cross-correlation), and (2) the predictability in the
181 pattern of turn-taking (% Markov fit), independent of the timing (*see supplemental information*).

182 In highly coordinated pairs, we expected that the delay of the male's (M) response to his
183 female (F) partner would be the same as that F's response to the M (comparison of M-F and F-M
184 calling delay). Furthermore, we expected higher consistency in the delay times between calls
185 (delay times of the M answer to the F, and the F answer to the M). These would be examples of
186 increased temporal coordination.

187 Previous work has shown visually isolated pairs are more Markovian in their alternation
188 of calls (Perez et al. 2015b). More Markovian patterns mean that the identity of a given caller
189 depends on the identity of the previous caller. In this way, the pattern of turn taking is assessed
190 independently of the calling delays. This phenomenon may be analogous to how speech patterns
191 in humans change between talkers when they are face-to-face in contrast to when they are on the
192 phone. More Markovian exchanges are likely a result of the birds' increased attention to the
193 acoustic channel (Perez et al. 2015b). Together, the cross-correlation and % Markov fit provide a
194 description of the pattern of intra-pair vocal dynamics.

195 Additionally, we randomly selected a subset of 50 calls from each recording for each
196 individual to use for the analysis of acoustic structure (function: "random", a uniform random
197 selection in python). These calls were then manually examined, and only cleanly recorded calls
198 were kept for further analysis (calls that did not include noises from wing/body movements, the
199 cage, or other vocalizations from the partner). On average there were 121 ± 32 (Mean \pm SD) calls
200 per individual per condition. Final sample sizes are: N=1991, Female CON; N=1608, Female
201 WR; N=1683, Male CON; and N=1670, Male WR. The majority of calls were 'stack' calls (Ter
202 Maat et al. 2014; Gill et al. 2015) (*see supplemental information*).

203 This subset of extracted calls was used to quantify the effect of water condition on the
204 acoustic structure of calls. We used two complementary methods of analysis: (1) full-spectrum

205 analysis (Mouterde et al. 2014a; Elie and Theunissen 2016), and (2) quantification of pre-defined
206 parameters of the spectral envelope (Seewave package (Sueur et al. 2008)) implemented in R
207 software (v. 3.2.3, 2015, R Foundation for Statistical computing).

208

209 *2.5 Analysis of acoustic structure*

210 We used a standard two-tier approach (full spectrum analysis and pre-defined acoustic
211 features) to describe the effects of water restriction on the acoustic structure of vocalisations. The
212 two approaches we used answer slightly different questions. First, we asked whether water
213 condition affected the acoustic structure of vocalisations in any way, i.e. “Is there any
214 difference?”. In order to comprehensively analyze the spectrogram, we used an in-house program
215 (written in R) to summarize all the information in the entire spectrogram by using all the data
216 points of the spectrograms as raw measures in a PCA (Mouterde et al. 2014b; Elie and
217 Theunissen 2016) (*see supplemental information*). The full spectrum analysis allows for an
218 unbiased assessment of how WR affected the spectrogram (there is no *a priori* selection of
219 specific acoustic elements); however, the principal components cannot be easily related back to
220 specific acoustic features. Thus, we also asked “are there acoustic differences in traditionally
221 defined parameters?”. For this analysis we used Seewave package in R (Sueur et al. 2008) to
222 quantify the effect of water condition on four pre-defined acoustic features: an estimate of call
223 loudness (root-mean-square RMS of the temporal envelope) and three parameters of the spectral
224 envelope (median freq (kHz), stDev (kHz), and IQR (kHz)) (*see supplemental information*).

225

226 *2.6 Circulating testosterone and corticosterone levels*

227 For each individual, blood samples were collected during both treatments after acoustic
228 recordings were taken. Note that this means there was a minimum of 2 weeks between blood
229 sample collection and a subsequent recording. Both partners of a pair were bled at the same time
230 by two experimenters (M.A.S.F and N.H.P). All blood samples were collected between 11:00-
231 13:00 h and within 3 min of opening the cage door to catch individuals (Mean \pm SEM: 55 \pm 6
232 sec). Blood samples were centrifuged to obtain plasma (10 min at 10,000 x g), which was stored
233 at -20°C until further processing. Circulating concentrations of steroids were assayed using
234 enzyme immunoassay kits (Cayman Chemical Co., Ann Arbor, MI: Testosterone, 582701;
235 Corticosterone, 500655) (*see supplemental information*).

236

237 2.7 Statistics

238 All statistical analyses were carried out in R (v. 3.2.3, 2015, R Foundation for Statistical
239 computing). We used generalized linear-mixed models (LMMs; function lmer from the lme4
240 Package). The design of our study included three potential factors, Water Condition (WR and
241 CON), Recording number (1-6: indicating the order recordings were made in), and Cohort
242 (Cohort 1, CON first and Cohort 2, WR first). We used Recording Nb as a continuous variable in
243 our models. We did not include Cohort as a factor. For pair-level dependent variables, Pair was
244 included as a random factor. For individual-level dependent variables, Individual was included as
245 a random factor, and Sex was included as a between-subjects factor. Prior to interpretation, we
246 checked the validity of each model by plotting the distribution of the residuals. Data were
247 transformed as necessary. Model summaries are provided in Supplementary Table 1-7.

248

249 2. Results

250 3.1 WR effects on circulating corticosterone and testosterone

251 Consistent with prior research, water restriction had no effect on circulating
252 corticosterone levels in males or females (*Males*, mean \pm SEM, CON: 1.64 ± 0.43 ng/mL; WR:
253 1.17 ± 0.14 ng/mL; $\chi^2(1) < 0.01$, $P=0.987$; *Females*, mean \pm SEM, CON: 1.34 ± 0.23 ng/mL;
254 WR: 1.43 ± 0.35 ng/mL; $\chi^2(1) = 1.48$, $P=0.225$). Additionally, circulating testosterone levels were
255 low for males regardless of water condition and there was no main effect of water restriction on
256 circulating testosterone (mean \pm SEM, CON: 0.41 ± 0.10 ng/mL; WR: 0.47 ± 0.15 ng/mL; $\chi^2(1)$
257 $=0.40$, $P=0.527$). The low circulating level of testosterone for males in both treatment groups
258 suggests that the effect of water restriction was **smaller in the current study** than what has been
259 previously reported (Prior et al. 2013).

260

261 3.2 WR decreases overall vocal activity

262 Overall, in response to water restriction, zebra finches vocalized less. This effect was
263 clearest on male song rate during water restriction ($\chi^2(1) = 6.70$, $P=0.009$) (Figure 3A). In
264 addition, there was a small, but statistically significant decrease in call rate (calculated from the
265 entire recordings) for both males and females in response to water restriction (*Females*, mean \pm
266 SEM, CON: 3.03 ± 0.43 calls/min; WR: 2.67 ± 0.29 calls/min. *Males*, mean \pm SEM, CON: 3.56
267 ± 0.57 calls/min; WR: 2.92 ± 0.34 calls/min) (Water Condition $\chi^2(1) = 6.66$, $P=0.010$. Sex $\chi^2(1)$
268 $= 0.87$, $P=0.350$. Recording nb $\chi^2(1) = 0.09$, $P=0.763$. Water Condition \times Sex $\chi^2(1) = 0.24$,
269 $P=0.877$).

270 As we were primarily interested in intra-pair calling behavior, we also quantified the
271 effect of water restriction on calling during 'bursts', **periods of elevated calling activity**. There
272 was no main effect of water restriction on burst rate (number of bursts/ hour of recording) (Water

273 Condition $\chi^2(1) = 0.65$, $P = 0.420$) (Figure 3B); however, as with call rate during the entire
274 recording, water restriction caused a decrease in male and female call rate within bursts (Water
275 Condition $\chi^2(1) = 4.53$, $P = 0.033$; Sex $\chi^2(1) = 0.01$, $P = 0.940$; Water Condition \times Sex $\chi^2(1) = 0.02$,
276 $P = 0.881$) (Figure 3C). Throughout the course of the experiment, the burst rate increased from an
277 average of 4.14 ± 0.31 bursts/h in recording 1 to 6.01 ± 0.47 bursts/h in recording 6 (mean \pm
278 SEM) (Recording nb $\chi^2(1) = 19.29$, $P < 0.001$); however, call rate within bursts did not change
279 over the course of the experiment (Recording nb $\chi^2(1) = 0.04$, $P = 0.850$).

280

281 *3.3 WR influenced song composition*

282 Water restriction did not change the duration of male full motifs (Water Condition, $\chi^2(1)$
283 $= 2.88$, $P = 0.090$) (Figure 4A), but significantly affected other global song elements. More
284 specifically, when water restricted, males produced songs with a decreased proportion of full
285 motifs (duration of full motifs/total song bout (s)) (Water Condition, $\chi^2(1) = 4.46$, $P = 0.037$)
286 (Figure 4B), an increased duration of inter-element intervals (Water Condition, $\chi^2(1) = 5.81$,
287 $P = 0.016$) (Figure 4C), and an increased proportion of introductory notes (duration of
288 introductory notes/ full motifs (s)) (Water Condition, $\chi^2(1) = 7.18$, $P = 0.007$) (Figure 4D).

289

290 *3.4 WR does not affect F-M calling dynamics*

291 In contrast to male song, there was no significant effect of water restriction on the
292 patterns of intra-pair calling (temporal coordination or turn taking). First, there was no effect of
293 water restriction on our measure of temporal coordination (cross correlation of F-M and M-F
294 calling delay) (Figure 5A). The cross-correlation demonstrates that there was consistent temporal
295 delay in M-F calling which was unaffected by water condition. Calls were answered in ~ 200 ms

296 (regardless of whether the male or female was answering). Additionally, there was no effect of
297 water restriction on our estimate of turn-taking within a pair (% Markov Fit) (Water Condition:
298 $\chi^2(1) = 3.03$, $P = 0.081$; Recording Nb: $\chi^2(1) = 0.07$, $P = 0.789$, Figure 5B).

299

300 *3.5 WR has sex specific and vocalisation specific effects on acoustic structure*

301 3.5.1 WR has effects on acoustic parameters of song elements

302 In order to describe the effect of Water Condition on the acoustic structure of songs, we
303 used two complementary analyses. From the full spectrum analysis, we found no effect of Water
304 Condition on song elements, but some effects on individual notes and song syllables (Table 1).
305 More specifically, for the last introductory note PC1 and PC2 were significantly affected by
306 water restriction. Additionally, for the early song syllable PC3 was affected by Water Condition.

307 Consistent with the full spectrum analysis, we saw effects of Water Condition on pre-
308 defined acoustic features. Again, there was no effect of Water Condition on full motifs (Table 1;
309 Table 2). There was a significant effect of Water Condition on amplitude and interquartile-range
310 (IQR) for partial motifs (IQR was lower during WR and RMS was higher: Table 1; Table 2).
311 Similar to our full spectrum analysis, we saw significant effects of Water Condition on the last
312 introductory note and early song syllable (Table 1; Table 2). More specifically, the last
313 introductory note was at a lower amplitude when produced during water restriction (Table 1;
314 Table 2). Additionally, the early song syllable had an increased IQR (Table 1; Table 2).

315

316 3.5.2 Sex specific effects of water restriction on the acoustic parameters of calls

317 As with song, we used two complementary methods to examine the effect of Water
318 Condition on the structure of calls (predominately short, stack-like calls). First, we conducted the

319 full spectrum analysis on all of the calls. There was a main effect of Water Condition on PC3
320 (Table 3). However, there was also a highly significant Water Condition \times Sex for PC1, PC2,
321 and PC3 (Table 3). Therefore, we also ran the full spectrum analysis on female and male calls
322 separately. From these results, we see a clear effect of Water Condition on female calls (PC1,
323 Table 3) and male calls (PC1 and PC2, Table 3).

324 To complement the full spectrum analysis, we also examined the effect of Water
325 Condition on specific pre-defined acoustic features. For each of our acoustic parameters (RMS,
326 median frequency, SD, and IQR), we found highly significant Water Condition \times Sex
327 interactions (Table 3 and Table 4). WR had different effects on males and females (Table 3). For
328 female calls, WR decreased amplitude of calls (Table 3, Table 4), but increased Median
329 Frequency (Table 3, Table 4), StDev (Table 3, Table 4), and IQR (Table 3, Table 4). In contrast,
330 WR increased the amplitude of male calls (Table 3, Table 4), while decreasing the Median
331 Frequency (Table 3, Table 4).

332

333

334

335 **3. Discussion**

336 There are widespread changes in both vocal production and auditory perception in
337 seasonally breeding species, which raises the question of whether auditory-vocal communication
338 in opportunistically breeding species is also affected by reproductive states and/or environmental
339 conditions (Bass et al. 2016; Alward et al. 2017b). Our results suggest that zebra finch vocal
340 production is affected by environmental condition. More specifically, we describe several effects
341 of water restriction on male and female vocal behavior: water restriction (1) decreased the vocal

342 activity of both males and females (calling and male song), (2) shifted the composition of male
343 song so that it more closely resembled undirected song, and (3) influenced the acoustic structure
344 of all vocalizations in sex- and vocalisation- specific ways which we discuss below. Based on
345 previous research, we would anticipate that these changes are discriminable and could be salient
346 to partners and other conspecifics. The fact that these effects are vocalization and sex specific
347 raises interesting questions about how WR is impacting the acoustic structure of vocalizations. In
348 addition to neuroendocrine changes associated with decreased breeding readiness, there may be
349 context-specific changes in socio-sexual motivation in response to a lower environmental state.
350 Assuming that these are acoustic indicators of breeding readiness, this raises the question of
351 whether this information is used by the partner and other conspecifics. In order to determine if
352 these effects are specific to the pair-directed behavior, it would be useful to identify the effects of
353 water restriction on vocal dynamics for other social relationships and social conditions as well.

354

355 *4.1 Effects of water restriction on vocal activity*

356 Seasonally breeding bird species experience profound neural and vocal plasticity related
357 to breeding season (Beecher and Brenowitz 2005). In our current study, the decrease in vocal
358 output is similar to the decrease in song output seen in temperate-breeding songbirds. This is not
359 altogether surprising considering zebra finches have similar shifts in neuroendocrine state
360 associated with breeding as seasonally-breeding species. For zebra finches, changes in
361 reproductive and or breeding state are associated with changes in circulating steroid levels
362 (Perfito et al. 2007; Prior et al. 2013; Gill et al. 2015; Prior et al. 2017), brain steroid levels (Prior
363 et al. 2013), and nuclei volume for brain regions associated with song production (HVC and area
364 X) (Perfito et al. 2015). While we did not see changes in circulating testosterone levels in males

365 here, we did not measure other circulating or brain steroids. Furthermore, in zebra finches, as in
366 seasonally-breeding birds, sex steroids can have significant effects on male song (Arnold 1975;
367 Prior et al. 2016). In temperate, seasonally-breeding species, the decrease in song output during
368 non-breeding periods is generally assumed to be the result of changes in behavioral context and
369 neuroendocrine condition, which together result in the absence of breeding-condition song
370 signals. Similarly, we interpret the effect of water restriction on song output in male zebra
371 finches to reflect the changes in neuroendocrine state induced by water restriction.

372 The relationship between calling behavior and singing is not well understood, and thus it
373 is particularly interesting that we find some evidence that water restriction decreases calling
374 activity in addition to song. This effect could be interpreted as a decrease in (1) general vocal
375 output, (2) attention or focus on the auditory channel, or (3) sociosexual motivation. There is
376 evidence in zebra finches that the same brain nuclei in the song control system that regulate
377 song, also regulate aspects of calling behavior (Ter Maat et al. 2014; Benichov et al. 2016).
378 Therefore, it is possible that the effect of water restriction on specific brain regions could
379 influence call and song output similarly. Another possibility is that the motivation to call and
380 sing are related. The fact that water restriction did not impact the quality of calling exchanges
381 between zebra finch mates suggests that water restriction did not have an impact on the quality of
382 the pair bond and/or motivation to stay bonded. This is consistent with what is known about pair
383 bonding in zebra finches (Zann 1996; Prior and Soma 2015). In this study, we chose to examine
384 behavior under a relatively controlled social context (visual separation) and to focus on pair
385 bonded birds. Therefore, it is unclear to what extent this effect of water restriction would extend
386 to vocal behavior in other social contexts. In seasonally-breeding song sparrows, for example,
387 there is a decrease in song output in the winter, yet males sing at the same rate during simulated

388 territorial intrusions (Maddison et al. 2012). In zebra finches, which are non-territorial and form
389 life-long pair bonds, it would be particularly interesting to determine whether calling activity is
390 similarly affected by water restriction within other social relationships (e.g. familiar and novel
391 birds) and social contexts (e.g. traveling, foraging, etc).

392

393 *4.2 Effect of water restriction on acoustic structure*

394 Several lines of more recent research have described plasticity in the acoustic structure of
395 calls depending on ontogeny (Villain et al. 2015), social context (Hile and Striedter 2000), and
396 internal physiological state (Perez et al. 2012; Perez et al. 2015a; Perez et al. 2016). Based on our
397 current knowledge of zebra finch perception, we have every reason to expect that zebra finches
398 can hear very small differences in the acoustic structure of their harmonic calls. Amongst birds,
399 zebra finches are particularly sensitive to small spectro-temporal changes in harmonic complexes
400 (Lohr and Dooling 1998; Dooling et al. 2002; Dooling and Lohr 2006; Lohr et al. 2006; Prior et
401 al. 2018), therefore, while the effects of water restriction that we report here are small (e.g. 100
402 hz in median frequency), it is reasonable to assume these small differences would be perceptible
403 to zebra finches.

404 One of the simplest explanations for seasonal plasticity in acoustic structure is that this
405 plasticity is due to changes in syrinx size mediated by circulating testosterone levels (Suthers et
406 al. 1999). However, the differences we report are unlikely to be related to shifts in syrinx size.
407 First, we did not see changes in circulating testosterone levels in the periphery. Furthermore, we
408 would expect that changes in syrinx volume would result in a change in fundamental frequency
409 across the entire song (Cynx et al. 2005; Alward et al. 2016). Alternatively, changes in brain sex
410 steroid levels alone within key nuclei of the song control system can impact spectral features of

411 song syllables (Alward et al. 2017a; Alward et al. 2017b). It is likely that zebra finches
412 experience significant changes in brain steroid levels independent of peripheral changes (Prior et
413 al. 2013), and it is possible, although it has not been tested, that there are also effects of WR on
414 other neuromodulatory systems as well as sex steroid receptors which could explain these results.
415 Here we only looked at stack-like calls and male song, thus it is unclear if these changes in
416 acoustic structure extend across call types. Regardless of the mechanism, the changes in acoustic
417 structure could serve as a functional cue of reproductive condition.

418

419 *4.3 Functional significance of flexibility of calls*

420 It may seem surprising that the effect of water restriction on calls differed between males
421 and females. The results of our full spectrum analysis suggest that WR has a significant effect of
422 water restriction on both male and female calls. However, for each of our pre-defined acoustic
423 features, the effect of water restriction was in the opposite direction for males and females.
424 Female water restricted calls were lower amplitude, higher frequency (median frequency) and
425 more variable (standard deviation and inter-quartile range). In contrast, male water restricted
426 calls were higher amplitude and had a lower frequency (median frequency). From an ecological
427 perspective, it makes sense that females may be more sensitive to environmental perturbations
428 (Perfito 2010; Prior et al. 2013), and it is possible that female reproductive condition may be a
429 bottleneck on the timing of breeding bouts. Furthermore, water restriction has a more profound
430 effect in females, including significantly reducing the size of the female ovary and oviduct. In
431 contrast, water restriction has only a small effect on male testis size (Prior et al. 2013; Perfito et
432 al. 2006). Although in the current study, the effects of water restriction on male song suggest that
433 there was a shift in the central neuroendocrine state of male zebra finches. Thus, one

434 interpretation of the sex differences in the effect of water restriction on calls is that water
435 restriction had differing effects on the neuroendocrine state of males and females. Further
436 research describing the effect of water restriction and reproductive readiness on vocal dynamics
437 across social conditions and contexts would help clarify what functional relevance this difference
438 may have.

439 Importantly, the stack-like call that we investigated here is not specific to nesting periods
440 (Gill et al. 2015). Thus, regardless of the mechanisms resulting in changes in acoustic structure,
441 we would expect that environmental and/or reproductive condition dependent flexibility in stack-
442 like calls would precede other early breeding behaviors such as identifying a nesting site and
443 building a nest. This raises the hypothesis that such vocal flexibility could play a functional role
444 in coordinating partners' breeding attempts.

445

446 *4.4 Functional significance of the effect of water restriction on male song*

447 This is not the first report suggesting that breeding condition affects male song in zebra
448 finches. Previous paradigms using water restriction have reported similar decreases. In Prior et
449 al. 2013 we did not find a statistically significant effect of water restriction on male song during
450 a partner separation and reunion paradigm; however, qualitatively males did spend less time
451 singing. Additionally, Rashotte et al. (2001) also reported a significant effect of brief (2 day)
452 water deprivation on song.

453 Beyond just a decrease in overall song output, we might also expect a change in the type
454 of song and/or performance. Zebra finches produce two types of song, directed and undirected,
455 which are composed of the same stereotyped motif but differ in more subtle aspects such as the
456 number of introductory notes and overall stereotypy of the performance (Sossinka and Böhner

457 1980). Performances of directed song, or courtship song, involve the male facing the female and
458 often involve other aspects of courtship dance (Zann 1996). In contrast, undirected song can be
459 performed anywhere, and the male appears to be singing to himself (Zann 1996). In our current
460 study, these pairs were always visually isolated. Therefore, all song produced under this
461 behavioral paradigm would technically be classified as undirected song. However, taken
462 together, the effects of water restriction on song that we report here are broadly consistent with a
463 shift from directed to undirected zebra finch song. More specifically, water restriction increased
464 inter-sequence interval and the proportion of introductory notes as would be expected if there
465 were a shift from directed to undirected song (Woolley and Doupe 2008; Rajan and Doupe
466 2013). Additionally, our full spectrum analysis revealed that water restriction had the most
467 profound effect on the last introductory note and the early song syllable, again similar to what
468 would be expected from undirected song (Rajan and Doupe 2013).

469 A key difference between undirected and directed song is the motivational state of the
470 male. Likewise, we would expect a similar shift in motivational state (away from
471 courtship/breeding behaviors) following water restriction. Together these results raise the
472 hypothesis that the changes in song are related to shifts in sexual motivation following a reduced
473 state of breeding readiness.

474

475 4. *Conclusion*

476 Our current results contribute to several lines of evidence suggesting there are widespread
477 seasonal changes in audio-vocal communication (Catchpole and Slater 2008; Bass et al. 2016).
478 Our work supports the notion that this vocal plasticity extends to non-seasonally-breeding
479 species as well as to calling behavior. Furthermore, as our data suggests there is vocal plasticity

480 in response to environmental conditions, this raises the question of whether information on
481 breeding-state is present within zebra finch vocalisations and how zebra finches may use this
482 information to coordinate breeding attempts.

483

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496

497 **Conflict of Interest.**

498 The authors have no conflict of interest to declare.

499

500 **Ethical Approval**

501 This work was conducted in accordance with the French and European animal care legislation
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504

505 **Informed Consent**

506 Not applicable.

507

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664 Table 1: Effect of water restriction on the first three components of the full spectrum analysis and pre-defined acoustic parameters.

		Full Spectrum						Pre-Defined acoustic parameters							
		Axis 1		Axis 2		Axis 3		RMS		Med (kHz)		SD (kHz)		IQR (kHz)	
		ChiSq	P	ChiSq	P	ChiSq	P	ChiSq	P	ChiSq	P	ChiSq	P	ChiSq	P
Global Song Elements	Full Motif	0.02	0.891	0.25	0.615	0.72	0.397	2.90	0.088	3.03	0.082	0.15	0.696	0.33	0.565
	Partial Motif	0.07	0.791	0.10	0.757	0.05	0.827	3.85	0.049	0.16	0.689	0.07	0.798	4.31	0.038
		Q1: Is there evidence of any difference? NO						Q2: Are there acoustic differences in traditionally defined parameters? Yes, statistically significant, but very small effect of WR on partial motifs							
Individual Notes	First IN	0.07	0.798	1.26	0.261	0.12	0.726	0.48	0.487	0.26	0.609	0.22	0.641	3.47	0.063
	Last IN	11.50	<0.001	2.46	0.117	6.67	0.010	5.68	0.017	0.51	0.477	1.71	0.191	0.36	0.546
	Early Song Syllable	2.36	0.125	1.71	0.191	4.87	0.027	3.08	0.080	1.11	0.293	2.14	0.143	3.85	0.049
	Late Song Syllable	1.26	0.261	2.17	0.141	0.27	0.605	0.05	0.831	2.50	0.114	2.78	0.096	0.79	0.373
		Q1: Is there evidence of any difference? YES in the last Introductory note and early song syllable						Q2: Are there acoustic differences in traditionally defined parameters? YES in the last Introductory note and early song syllable							

665 Note: To assess the effect of water restriction on the acoustic structure of male song we used mixed linear models (e.g. $lmer(acoustic$
666 $parameter \sim Water\ Condition + (1|Ind))$). Because so few males sang, we only included water condition (not Recording nb) in these
667 models. Bolded values indicate statistical significance ($P < 0.05$). $DF = 1$ for water condition.

668 Table 2: Effect of water restriction (Mean \pm SEM) on pre-defined acoustic parameters of song.

669

		RMS		Med (kHz)		SD (kHz)		IQR (kHz)	
		CON	WR	CON	WR	CON	WR	CON	WR
Global Song Elements	Full Motif	6.15 \pm 0.08	6.07 \pm 0.16	3.76 \pm 0.03	3.82 \pm 0.04	1.67 \pm 0.01	1.64 \pm 0.01	2.41 \pm 0.03	2.34 \pm 0.04
	Partial Motif	6.32 \pm 0.22	6.42 \pm 0.32	3.80 \pm 0.06	3.80 \pm 0.08	1.74 \pm 0.03	1.73 \pm 0.03	2.59 \pm 0.07	2.55 \pm 0.09
Individual Notes	First IN	10.14 \pm 0.68	9.22 \pm 0.37	2.66 \pm 0.09	3.10 \pm 0.16	1.89 \pm 0.03	1.92 \pm 0.05	2.63 \pm 0.13	3.09 \pm 0.13
	Last IN	10.41 \pm 0.21	9.29 \pm 0.41	2.71 \pm 0.08	3.06 \pm 0.17	1.81 \pm 0.04	1.94 \pm 0.04	2.59 \pm 0.13	2.93 \pm 0.13
	Early Song Syllable	10.85 \pm 0.29	9.78 \pm 0.38	3.25 \pm 0.06	3.25 \pm 0.06	1.80 \pm 0.04	1.87 \pm 0.04	2.6 \pm 0.12	2.83 \pm 0.11
	Late Song Syllable	10.27 \pm 0.24	10.89 \pm 0.37	3.65 \pm 0.06	3.79 \pm 0.06	1.49 \pm 0.02	1.49 \pm 0.03	2.06 \pm 0.07	2.83 \pm 0.10

670

671 Note: Bolded values indicate statistical significance (P<0.05).

672 Table 3. Effect of water restriction, sex, and recording number on the first three components of the full spectrum analysis and pre-
 673 defined acoustic parameters.

		Full Spectrum						Pre-Defined acoustic parameters							
		Axis 1		Axis 2		Axis 3		RMS		Med (kHz)		SD (kHz)		IQR (kHz)	
	Factor	ChiSq	P	ChiSq	P	ChiSq	P	ChiSq	P	ChiSq	P	ChiSq	P	ChiSq	P
All Calls	Water Cond	0.31	0.577	0.61	0.435	11.93	<0.001	2.13	0.144	13.21	<0.001	1.20	0.273	13.74	<0.001
	Sex	2.38	0.123	2.80	0.094	0.95	0.331	0.06	0.803	0.04	0.847	0.74	0.390	0.69	0.407
	Record. Nb	0.93	0.334	9.72	0.002	2.11	0.146	7.58	0.006	28.30	<0.001	1.98	0.160	0.02	0.884
	Water Cond:Sex	3.36	0.059	5.58	<0.001	13.93	<0.001	73.00	<0.001	112.10	<0.001	11.40	<0.001	26.90	<0.001
		Q1: Is there evidence of any acoustic difference? YES (Water Condition X Sex)						Q2: Are there acoustic differences in traditionally defined parameters? YES (for all four parameters there is a Water Condition X Sex interaction)							
Female Calls	Water Cond	14.68	<0.001	0.92	0.338	0.03	0.854	52.42	<0.001	121.90	<0.001	12.10	<0.001	44.61	<0.001
	Record. Nb	55.97	<0.001	1.67	0.196	0.007	0.935	21.80	<0.001	46.80	<0.001	7.73	<0.001	1.78	0.180
		Q1: Is there evidence of any acoustic difference? YES						Q2: Are there acoustic differences in traditionally defined parameters? YES (all four parameters)							
Male Calls	Water Cond	4.91	0.027	4.98	0.026	1.70	0.195	30.36	<0.001	24.74	<0.001	3.15	0.080	1.41	0.236
	Record. Nb	11.16	<0.001	7.64	0.006	7.23	0.007	0.80	0.371	1.07	0.301	0.37	0.542	2.11	0.150
		Q1: Is there evidence of any acoustic difference? YES						Q2: Are there acoustic differences in traditionally defined parameters? YES (RMS and median freq)							

674 Note: To assess the effect of water restriction on the acoustic structure of calls we used mixed linear models (e.g. ALL CALLS:
 675 lmer(acoustic parameter ~ Water Condition*Sex + scale(Recording nb) + (1|Ind)). CALLS BY SEX: lmer(acoustic parameter ~ Water

676 Condition + scale(Recording nb) + (1|Ind)). Bolded values indicate statistical significance ($P < 0.05$). DF=1 for water condition, sex
677 and recording number.

678 Table 4. Effect of water restriction (Mean \pm SEM) on pre-defined frequency parameters of male and female calls.

679

	RMS		Med (kHz)		SD (kHz)		IQR (kHz)	
	CON	WR	CON	WR	CON	WR	CON	WR
Female Calls	10.31 \pm 0.05	9.91 \pm 0.06	3.03 \pm 0.01	3.15 \pm 0.01	1.82 \pm 0.005	1.83 \pm 0.005	2.56 \pm 0.02	2.69 \pm 0.02
Male Calls	10.01 \pm 0.05	10.44 \pm 0.06	3.12 \pm 0.03	3.02 \pm 0.01	1.86 \pm 0.005	1.85 \pm 0.005	2.73 \pm 0.02	2.70 \pm 0.02

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685 es indicate statistical difference (P<0.05) between CON and WR conditions.

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688 Figure Legends:

689 Figure 1: Schematic of our general experimental paradigm. The vocal behavior of established
690 zebra finch pairs was recorded under two environmental conditions, water *ad lib* (Control) and
691 water restricted (WR). Experimental water restriction is known to reduce breeding readiness in
692 zebra finches. In order to focus on vocal behavior specifically, independent of other
693 communication channels, recordings were made while pairs were visually isolated. The table
694 summarizes the parameters of vocal behavior we used as dependent variables and the analyses
695 conducted. Briefly, timestamped calls and male song were extracted, and we used these
696 vocalisations to quantify three characteristics of vocal activity (male song rate, burst rate, and
697 call rate). Additionally, we quantified the effect of water restriction on the composition of male
698 song (e.g. global song elements such as introductory note sequence, full motifs and partial
699 motifs) and on the quality of the partners calling exchanges (cross-correlation of male-female
700 calls, and % Markov Fit of patterns of turn taking). Finally, we used two methods (full spectrum
701 analysis and pre-defined acoustic features) to assess the effects of water restriction on the
702 acoustic structure of vocalisations.

703

704 Figure 2: Spectrogram of a male song bout. Examples of extracted song elements are labeled:
705 (A) global song elements, and (B) individual syllables.

706

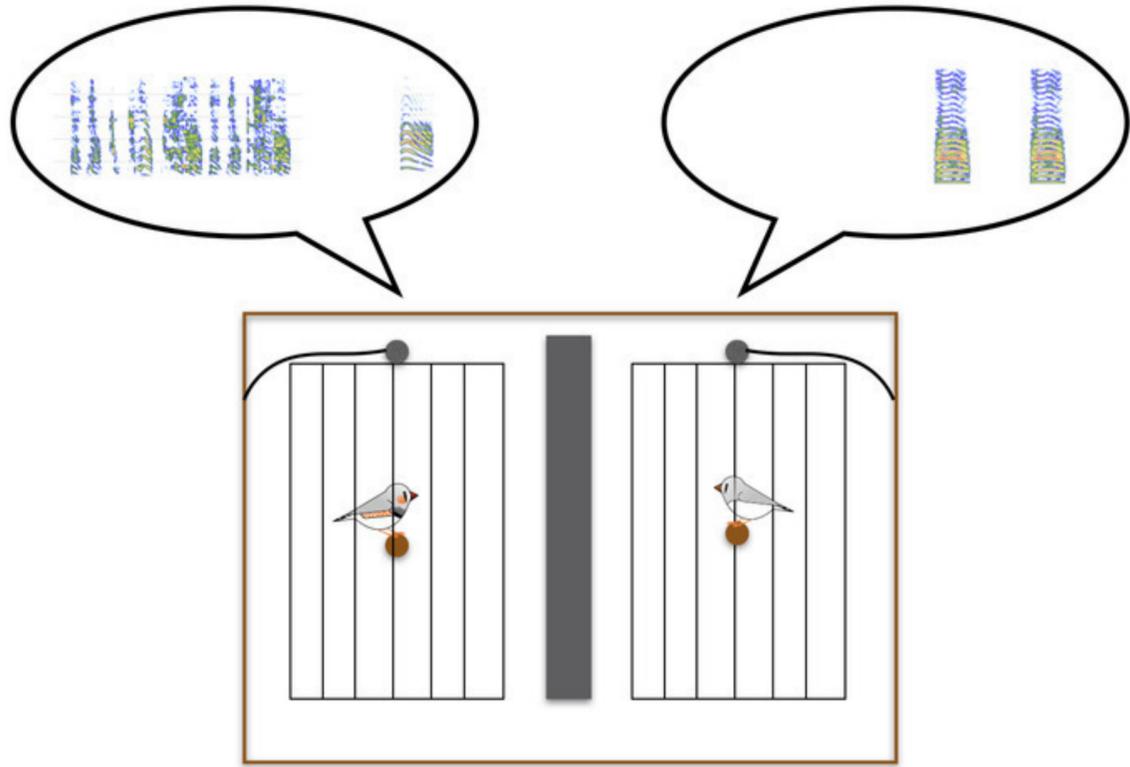
707 Figure 3: Effect of water restriction on vocal activity: (A) song rate (per hour). **Note that only**
708 **singing males are shown here.** (B) Burst rate (per hour). **Bursts are periods of elevated calling**
709 **activity.** (C) Individual call rate within bursts (per hour). **Call rate is plotted per hour to be**
710 **consistent with panels A and B. Call rate per min are as follows: Females, mean \pm SEM, CON:**

711 1.00 ± 0.25 calls/min; WR: 0.49 ± 0.09 calls/min. *Males*, mean \pm SEM, CON: 1.08 ± 0.29
712 1.08 ± 0.29 calls/min; WR: 0.60 ± 0.15 calls/min). Mean \pm Standard Error. * P <0.05, ** P <0.01, CON=
713 control (*blue circles*), WR=water restricted (*gray circles*). Circle = individual level parameter;
714 Hexagon = pair level parameter. Note that in Panel A, the height of the standard error bars is
715 equal to the height of the symbol for WR, therefore Prism does not include error bars in this
716 instance.

717
718 Figure 4: Effect of water restriction on song composition (A) duration of full motifs (s) (B) ratio
719 of full motifs to total duration of song bout, (C) duration of inter-sequence interval (s), and (D)
720 ratio of duration of introductory note sequence to duration of full motifs. Mean \pm Standard Error.
721 If error bars are not shown, they are smaller than the circle indicating the mean. * P <0.05, **
722 P<0.01. CON= control (*blue circles*), WR=water restricted (*gray circles*).

723
724 Figure 5: Effect of water restriction on coordination of calling exchanges. (A) Coordination
725 estimated by the cross-correlation of F-M calls showed no difference between exchanges under
726 WR and Con conditions. Mean \pm Standard Error. M-F: labels the female's delay to answer her
727 male partner. F-M: labels the male's delay to answer his female partner. (B) Effect of water
728 restriction estimation of turn taking, Markov fit; Mean \pm Standard Error. CON= control (*blue*
729 *hexagon*), WR=water restricted (*gray hexagon*).

Experimental Set Up



 *Water ad libitum*

&

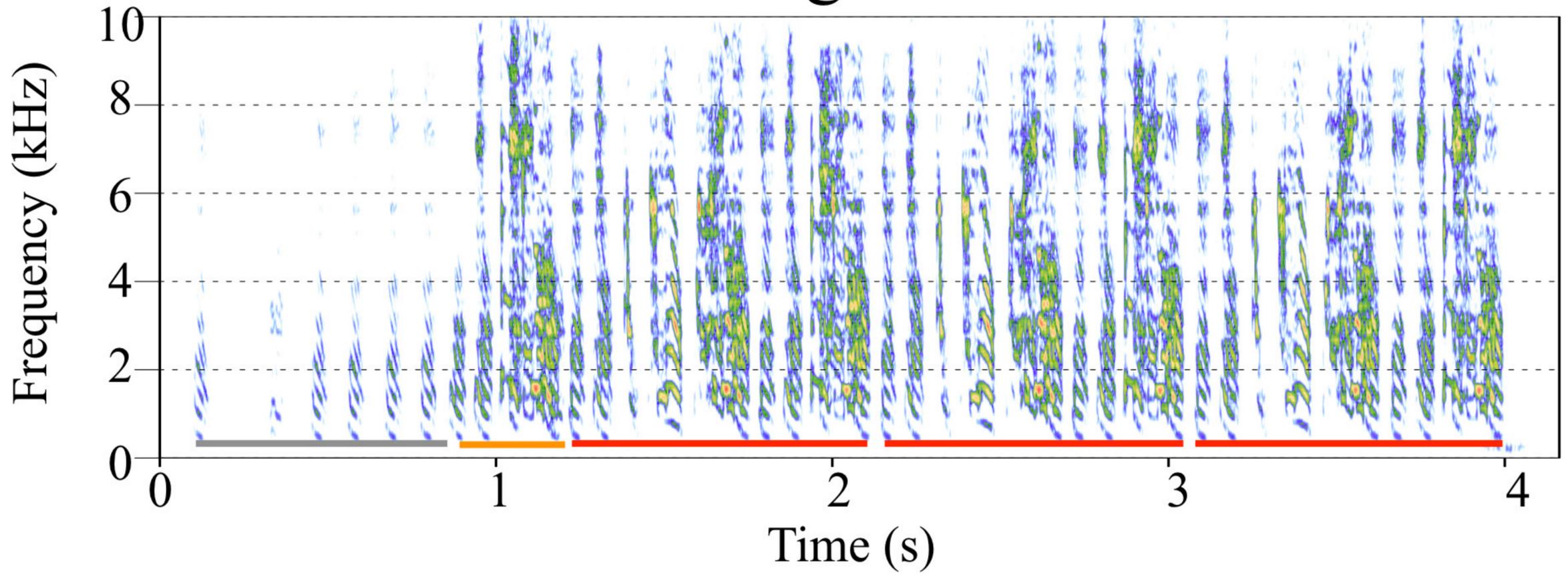
 *Water Restriction*

Summary of Dependent Variables and Analyses

Type	Dependent Variable	Notes on Analyses
Vocal Output	Song Rate	All songs were used.
	Call Rate	All calls were used.
	Burst Rate	Partner-directed calling appears to predominately occur during elevated calling events which we have termed bursts.
	Call Rate in Bursts	
Global Song Elements/Composition	Duration of Full Motifs	All songs were used to quantify the proportion of song bouts composed of the global song elements (introductory note sequence, full motifs and partial motifs).
	Proportion of Song Full Motifs	
	Inter-Sequence Interval	
	Proportion Introductory Notes	
Quality of Calling Exchanges	Male-Female Calling Delay	All calls, within bursts, were used to calculate the cross-correlation M-F calling delay.
	Male-Female Turn Taking	All calls, within bursts, were used to calculate % Markov Fit.
Acoustic Structure	Male and Female Calls	All song and a subset of good quality calls were used. Acoustic structure was assessed with two methods. First, all the data points of the spectrogram were used as raw measures in a PCA. Second, traditionally defined acoustic features (RMS, median freq, standard deviation and interquartile range of the spectral envelope) were quantified.
	Global Song Elements	
	4 Individual Notes Extracted from Song	

A

Song Bout



Extracted Sequences

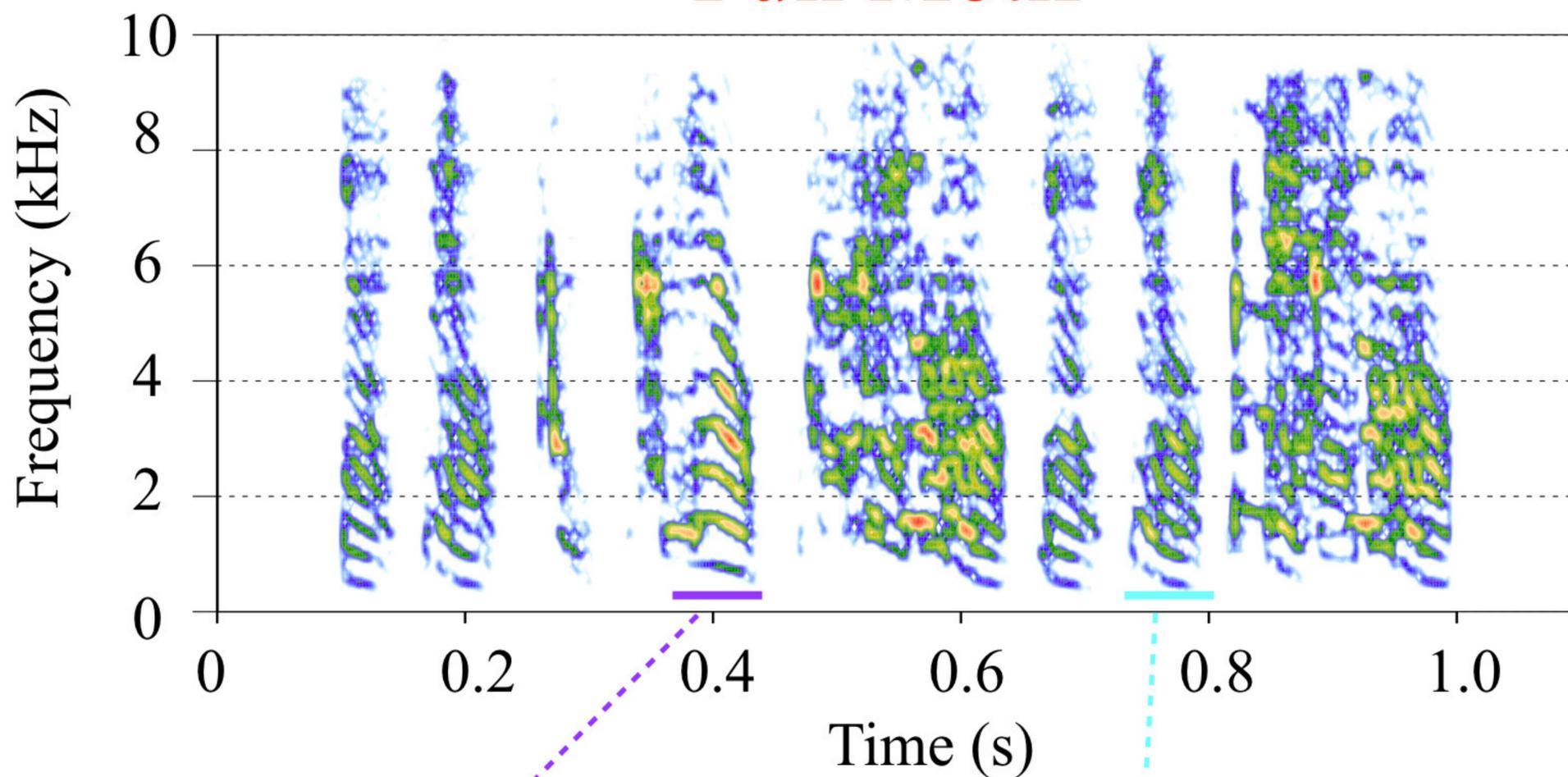
a) Introductory Notes

b) **Partial Motifs**

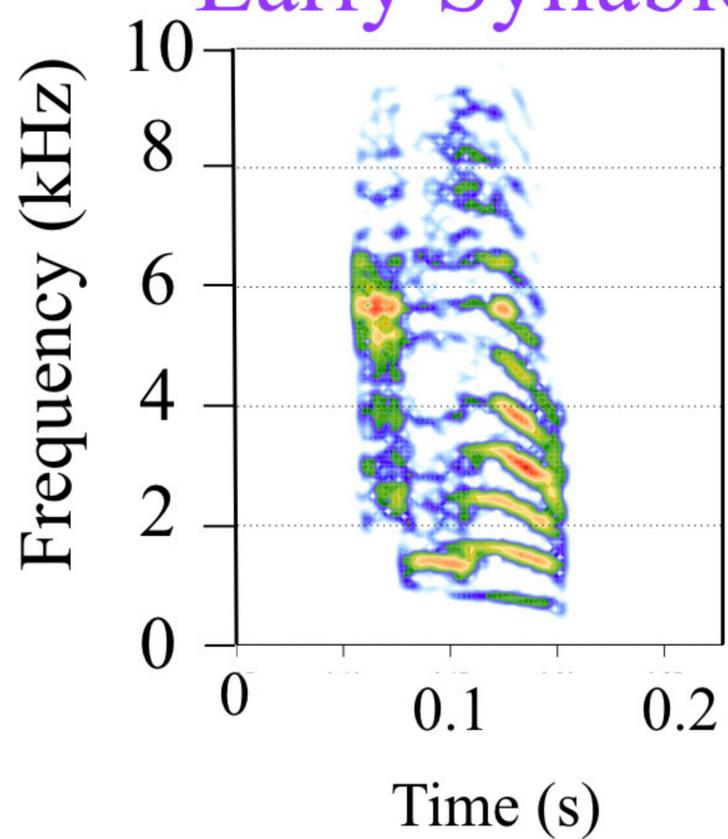
c) **Full Motifs**

B

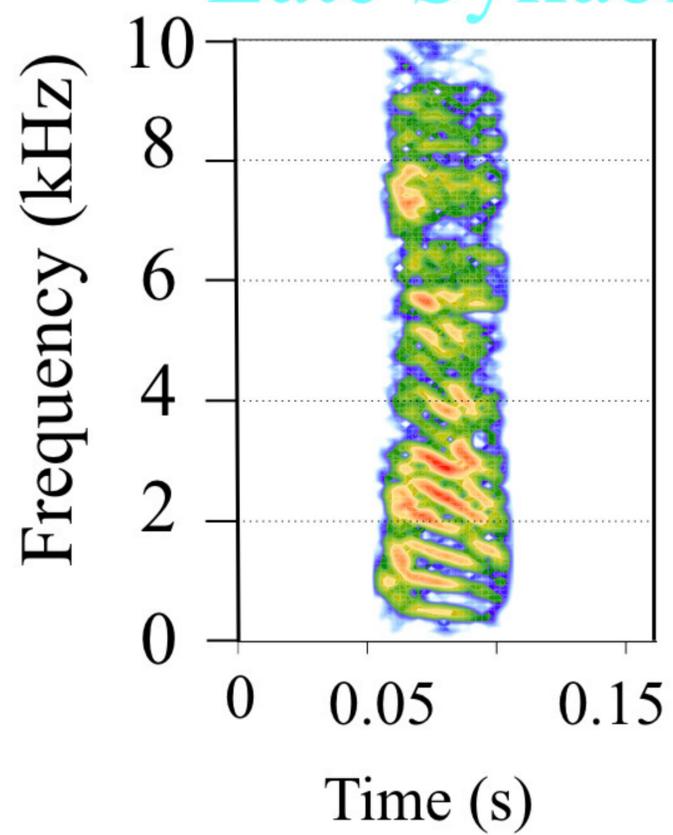
Full Motif

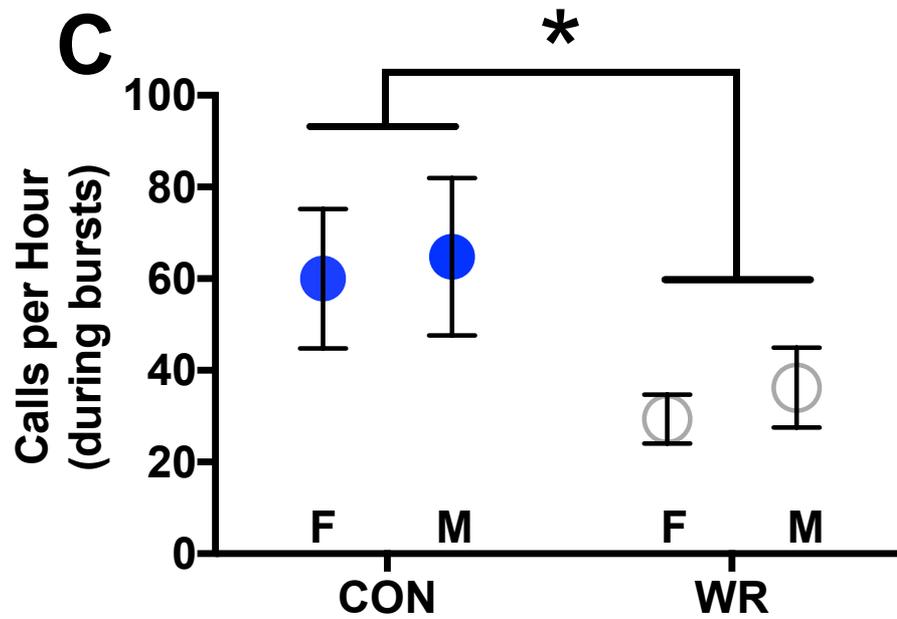
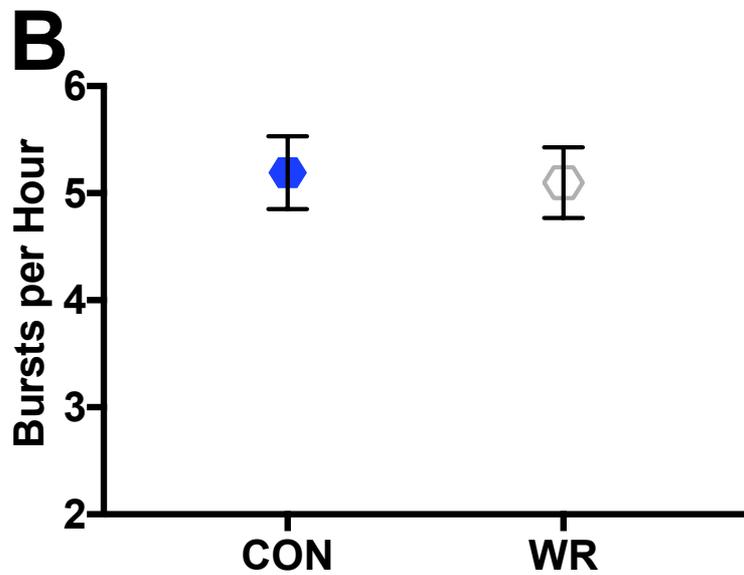
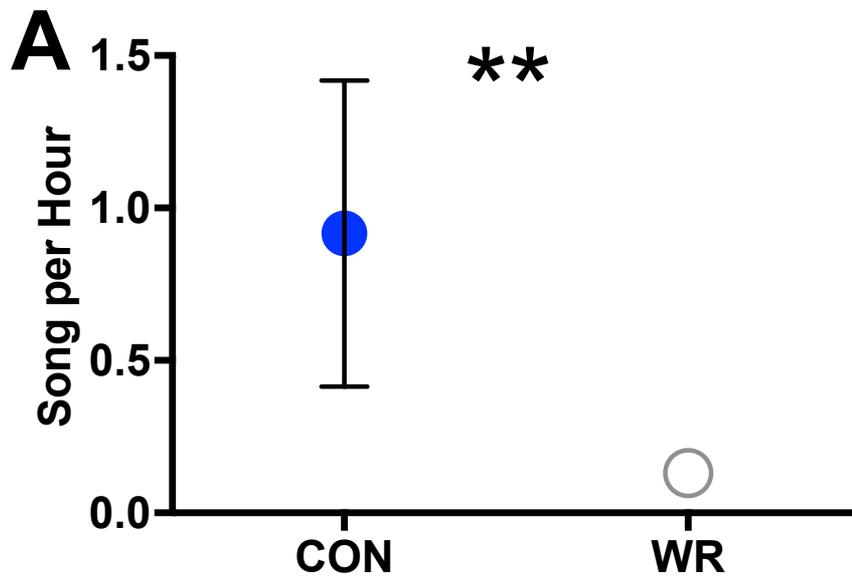


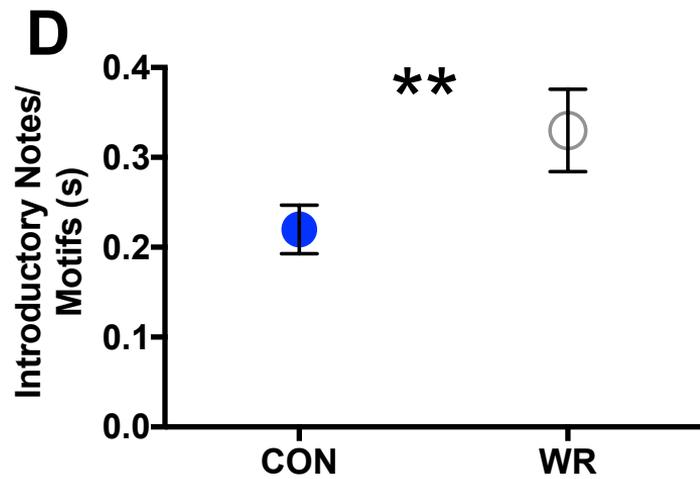
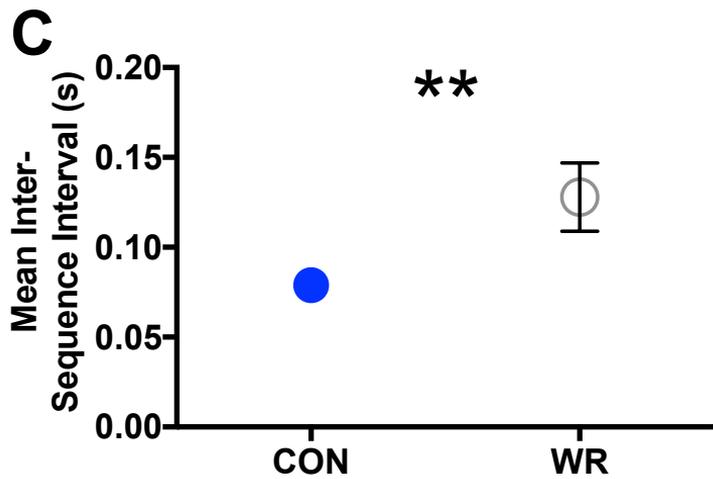
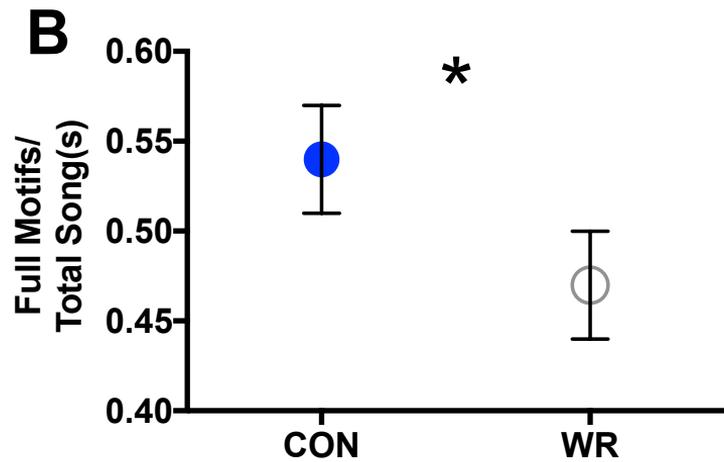
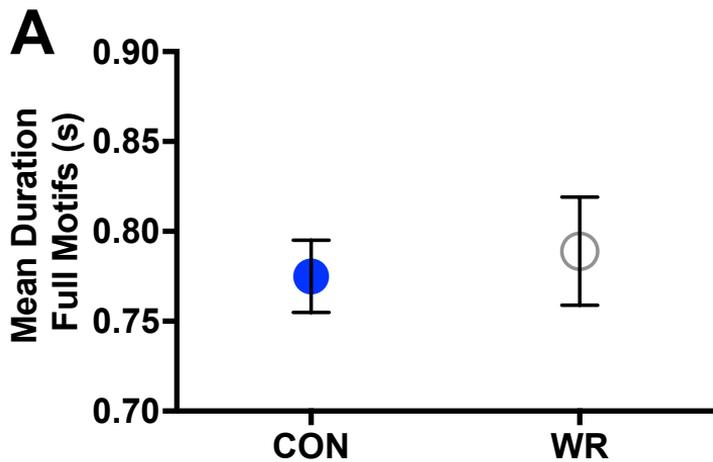
Early Syllable

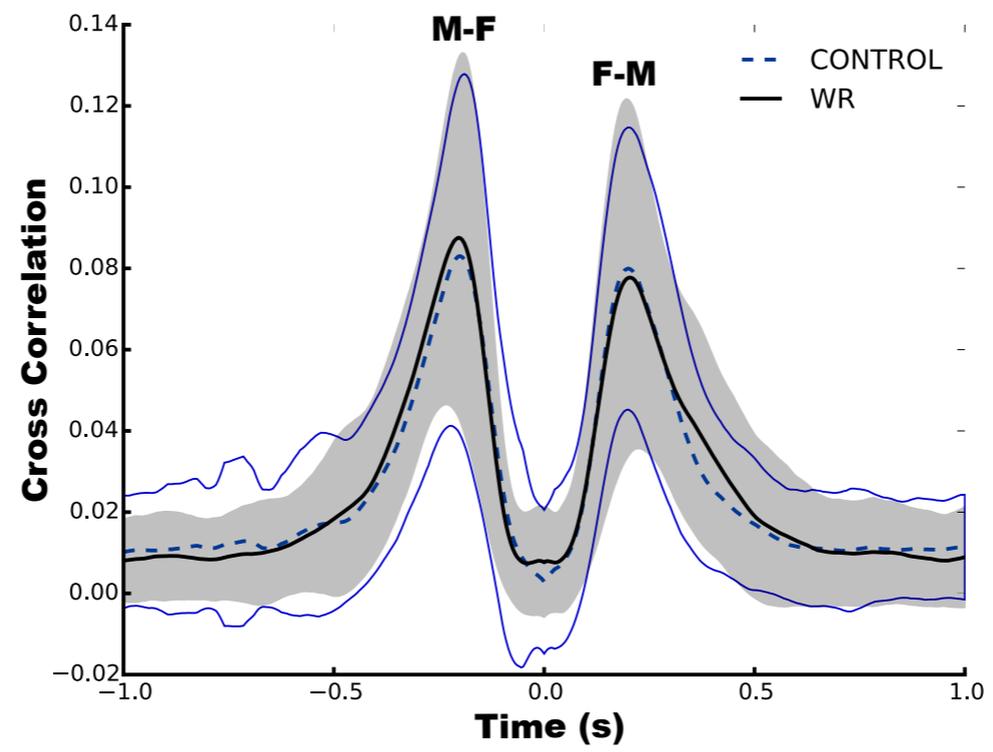


Late Syllable







A**B**