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7 ZENK expression in the auditory pathway of black-capped chickadees (*Poecile atricapillus*) as a
8 function of D note number and duty cycle of *chick-a-dee* calls

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Abstract

Black-capped chickadees (*Poecile atricapillus*) use their namesake *chick-a-dee* call for multiple functions, altering the features of the call depending on context. For example, duty cycle (the proportion of time filled by vocalizations) and fine structure traits (e.g., number of D notes) can encode contextual factors, such as predator size and food quality. Wilson and Mennill (2011) found that chickadees show stronger behavioral responses to playback of *chick-a-dee* calls with higher duty cycles, but not to the number of D notes. That is, independent of the number of D notes in a call, but dependent on the overall proportion of time filled with vocalization, birds responded more to higher duty cycle playback compared to lower duty cycle playback. Here we presented chickadees with *chick-a-dee* calls that contained either two D (referred to hereafter as 2 D) notes with a low duty cycle, 2 D notes with a high duty cycle, 10 D notes with a high duty cycle, or 2 D notes with a high duty cycle but played in reverse (a non-signaling control). We then measured ZENK expression in the auditory nuclei where perceptual discrimination is thought to occur. Based on the behavioral results of Wilson and Mennill (2011), we predicted we would observe the highest ZENK expression in response to forward-playing calls with high duty cycles; we predicted we would observe no significant difference in ZENK expression between forward-playing high duty cycle playbacks (2 D or 10 D). We found no significant difference between forward-playing 2 D and 10 D high duty cycle playbacks. However, contrary to our predictions, we did not find any effects of altering the duty cycle or note number presented.

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55 1. Introduction

56 Songbirds possess a unique vocal organ, the syrinx, that allows them to communicate
57 with individuals of both their own and other species using vocalizations of varying complexity
58 (Gill, 2007). Changes in the structural patterns of these vocalizations are easily noticeable by
59 songbirds, and do not need to be taught (Reber et al., 2016). *Chick-a-dee* calls, produced by
60 multiple Paridae species, including black-capped chickadees (*Poecile atricapillus*), are used to
61 convey a variety of information, such as threat posed by predators (Templeton, Greene, & Davis,
62 2005), recruitment to food sources (Mahurin & Freeberg, 2009), recruitment of conspecifics and
63 heterospecifics to mob a perched predator (Ficken & Witkin, 1977), as well as species-specific
64 information (Charrier & Sturdy, 2005). Chickadees are a popular model species used for
65 exploring the mechanisms behind information coding in acoustic signals, due to the complexity
66 and relative sophistication of *chick-a-dee* calls (see Wilson & Mennill, 2011).

67 *Chick-a-dee* calls are comprised of four main note types (A, B, C, and D notes), and they
68 follow a basic set of syntactical rules (see Figure 1). Note types may be duplicated or omitted in
69 a single call, though the notes will always follow the A> B>C>D order. Depending on the
70 acoustic structure of the call, different information can be encoded by a signaler and
71 subsequently decoded by a receiver. The signalers can encode information using several different
72 mechanisms, including alterations in sequence-level parameters (e.g., duty cycle; the proportion
73 of time that a bout of calls relative to inter-note silences occur in a vocalization), and structure
74 (e.g., note type, note frequency) of the call (Wilson & Mennill, 2011).

75 Previous research has examined the vocal and behavioral responses of chickadees hearing
76 *chick-a-dee* calls of varying acoustic structure. For example, Templeton, Greene, and Davis
77 (2005) demonstrated that, in general, black-capped chickadees produce mobbing calls containing

78 more D notes in response to the presence of smaller, more agile, high-threat predators (compared
79 to larger, less agile, low-threat predators). This suggests that number of D notes conveys the
80 degree of threat posed by predators. In contrast, Wilson and Mennill (2011) demonstrated that
81 the duty cycle (i.e., the proportion of time that a call can be heard) of *chick-a-dee* calls, not the
82 signal structure (e.g., note composition in the call), dictates the level of behavioral response by
83 conspecifics to playback of *chick-a-dee* calls; playback with high duty cycles attracted more
84 conspecific receivers, elicited quicker and closer approaches, and responding birds remained
85 within 10m of the playback speaker for longer than playback with low duty cycle. Furthermore,
86 they found that a receiver's behavioral response did not differ as a function of the number of D
87 notes; responses to both high duty cycle playback of calls with few D notes and high duty cycle
88 playback of calls with many D notes were statistically indistinguishable, suggesting that duty
89 cycle, not the number of D notes, is the salient feature (see Wilson & Mennill, 2011).

90 While variations in call properties have been demonstrated to elicit differential behavioral
91 responses such as the number of conspecific receivers attracted, as well as the rate of approach
92 by receivers (Wilson & Mennill, 2011), changes in call properties have also been found to lead to
93 differential amounts of immediate early gene (IEG) expression in Parid auditory areas. These
94 varied neural responses signify neural plasticity and altered perception in response to a changing
95 auditory environment. For example, it has been shown that *chick-a-dee* mobbing calls in
96 response to high threat predators have a corresponding higher expression of the IEG *Zif268/Egr-*
97 *1/NGFI-A/Krox-24 (ZENK)* in telencephalic auditory areas [i.e., caudomedial mesopallium
98 (CMM) and caudomedial nidopallium (NCM); see Avey et al., 2011]. Therefore, expression of
99 IEG such as ZENK in the auditory areas may provide insight into how receivers perceive
100 differences in duty cycle and call structure.

101 In the current study, we examined the amount of ZENK expression in the telencephalic
102 auditory areas of black-capped chickadees prompted by auditory playback of variations of *chick-*
103 *a-dee* calls, specifically variation in fine structure (i.e., number of D notes) and sequence-level
104 parameters (i.e., duty cycle). Based on previous neurobiological (Avey et al., 2011) and
105 behavioral results (Wilson & Mennill, 2011) our primary aim was to explore the independent and
106 combined effects of variation in call structure and variation in duty cycle on IEG expression.
107 Using male chickadees, we conducted a playback experiment with four conditions varying in
108 both duty cycle and number of D notes (Figure 2): (1) *chick-a-dee* calls containing 2 D notes
109 with a low duty cycle, (2) *chick-a-dee* calls containing 2 D notes with a high duty cycle, (3)
110 *chick-a-dee* calls containing 10 D notes with a high duty cycle, and (4) *chick-a-dee* calls
111 containing 2 D notes with a high duty cycle but played in reverse, thereby creating a non-
112 biologically-relevant stimulus and serving as a negative control (as in Avey et al., 2011). The
113 duty cycle was identical between the 2 D note and 10 D note high duty cycle groups, so any
114 differences in IEG expression would be due to perceptual differences in response to the number
115 of D notes. Similarly, the 2 D note high duty cycle and low duty cycle groups had identical call
116 structure, so any differences would be due to perceptual differences in response to duty cycle.

117 Based on Wilson and Mennill's (2011) results, we predicted that the highest levels of
118 ZENK expression would be found following playback of *chick-a-dee* calls with high duty cycles;
119 specifically, we predicted that *chick-a-dee* calls containing 2 D notes with a high duty cycle and
120 *chick-a-dee* calls containing 10 D notes with a high duty cycle would elicit similar levels of
121 ZENK expression.

122 **2. Methods**

123 **2.1 Subjects**

124 Twenty male black-capped chickadees caught from three sites in Edmonton, Alberta,
125 Canada (North Saskatchewan River Valley, 53.53N, 113.53W; Mill Creek Ravine, 53.52N,
126 113.47W; Stony Plain, 53.46N, 114.01W) were used in this study. All birds were captured
127 between 24 December 2010 and 26 January 2013, and were at least one year of age when
128 captured (identified by examining the color and shape of the rectrices; Meigs, Smith, & Van
129 Buskirk, 1983; Pyle, Howell, & Ruck, 1997). Post-capture, birds were housed indoors in
130 individual Jupiter Parakeet cages (30 × 40 × 40 cm, Rolf C. Hagen Inc, Montreal, QB, Canada)
131 that enabled visual and auditory, but not physical, contact with other male and female black-
132 capped chickadees. Colony rooms were kept on the natural light cycle of Edmonton, and
133 maintained at 20 degrees Celsius. Subjects were given *ad libitum* access to food (Mazuri Small
134 Bird Maintenance Diet; Mazuri, St. Louis, MO, U.S.A), water, grit, cuttlebone, and various
135 environmental enrichment materials (perches, separators, houses). A mixture of egg and spinach
136 or parsley, worms, and water supplements (Prime Vitamin Supplement; Hagen, Inc.) were given
137 on alternating days.

138 **2.2 Playback Stimuli**

139 Our playback stimuli were a subset of the *chick-a-dee* calls with varying duty cycles
140 and/or number of D notes that were originally constructed and used by Wilson and Mennill
141 (2011). Briefly, calls were obtained from a variety of sources, produced by several individual
142 chickadees, and were edited to create playback stimuli that were either low duty cycle with 2 D
143 notes or high duty cycle with either 2 D or 10 D notes. The 2 D high duty cycle stimuli and the
144 10 D note high duty cycle stimuli had identical duty cycles, to test the effect of fine structure
145 (i.e., number of D notes) rather than duty cycle. Calls were modified to contain a certain number
146 of notes, but each call contained notes produced by a single individual (see Wilson & Mennill,

2011 for additional details). Subjects were randomly assigned to one of four groups, with five birds per group, and each group being exposed to one of four types of acoustic stimuli: *chick-a-dee* calls with 2 D notes and a low duty cycle, *chick-a-dee* calls with 2 D notes and a high duty cycle, *chick-a-dee* calls with 10 D notes and a high duty cycle, or *chick-a-dee* calls with 2 D notes and a high duty cycle played in reverse. Stimuli consisted of two calls each produced by a different individual. It should be noted that during the *chick-a-dee* calls with 2 D notes and a high duty cycle, there are a greater number of 2-D note calls compared to the number of 10-D note calls during the *chick-a-dee* calls with 10 D notes and a high duty cycle (see Figure 2). In order to avoid pseudoreplication, each bird was presented with different calls (see Kroodsma et al., 2011 for additional details).

2.3 Playback procedure and equipment

Approximately 24 hours before playback, each bird was housed in a cage (Jupiter Parakeet), with access to food and water, in individual soundproof chambers (1.7m x 0.84m x 0.58m; Industrial Acoustics Corporation, Bronx, New York, USA) maintained on the natural summer light cycle of Edmonton, Alberta. All birds were exposed to the playback stimulus once a minute, repeated over 30 minutes. After this 30 minutes, birds were exposed to an hour of silence in the dark and then perfused immediately to ensure maximum quantity and quality of ZENK preservation (Mello & Clayton, 1994). A lethal dose of 0.04 ml of 100 mg/ml ketamine and 20 mg/ml xylazine (1:1) was administered intramuscularly to each subject. The bird was perfused via the left ventricle using heparinized 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA). The brain of each black-capped chickadee was then extracted and placed in a PFA solution for 24 hours, followed by a 30% sucrose PBS solution for 48 hours. The brains were then fast frozen using isopentane and dry ice and stored at -80°C until sectioned.

170 **2.4 Histology**

171 Brains were sectioned sagittally from the midline, and 48 40µm sections of each
172 hemisphere were collected and stored in PBS. In order to visualize ZENK, sections were first
173 washed twice in 0.1 M PBS for a minimum of five minutes, transferred to a 0.5% H₂O₂ solution
174 and incubated for 15 minutes. Incubation was followed by three 5 min washes in 0.1 M PBS. A
175 second incubation in 10% normal goat serum for 20 hours at room temperature followed.
176 Sections were then transferred into the primary antibody (erg-1, catalogue # sc-189, Santa Cruz
177 Biotechnology, Santa Cruz, CA, USA) for 24 hours at a concentration of 1: 5,000 in 0.1 M PBS
178 with Triton X-100 (PSB/T), then washed three times in PBS/T before being incubated in 1:200
179 biotinylated goat-anti-rabbit antibody (Vector Labs, Burlington, ON, Canada) in PBS/T for one
180 hour. After three more washes in PBS/T, sections were incubated in avidin-biotin horseradish
181 peroxidase (ABC Vectastain Elite Kit; Vector Labs, Burlington, ON, Canada) for one hour,
182 followed by three washes in 0.1M PBS. Sections were then processed with 3,3'-
183 diaminobenzidine tetrachloride (Sigma FastDAB, D4418, Sigma-Aldrich, Santa Fe Springs, CA,
184 USA) to visualize expression of ZENK, followed by three washes with 0.1M PBS to remove any
185 excess visualizing agents.

186 **2.5 Imaging**

187 Eight sections per individual were mounted on each slide and coverslipped. The first
188 eight medial sections in which the regions of interest were identified and contiguous (i.e.,
189 attached) to the telencephalon were used for imaging. Three neuroanatomical regions (CMM,
190 NCMd (dorsal), and NCMv (ventral)) were subsequently imaged using a Leica microscope
191 (DM5500B; Wetzlar, Germany) to quantify ZENK expression. Eight images of each region of
192 interest were captured per hemisphere, for a total of 48 images per subject. Images were obtained

193 using a 40x objective lens, a Retiga Exi camera (Qimaging, Surrey, BC, Canada), and Openlab
194 5.1 on a Macintosh OS X (Version 10.4.11). To ensure that each area was imaged in the same
195 location across slices and brains, we captured one image at each location as described in Avey et
196 al. (2008). Overlap in the ventral and dorsal regions of the NCM was carefully avoided by
197 imaging the ventral-most and dorsal-most portions as there are no distinguishing landmarks
198 between the two areas (Avey et al., 2014). ImageJ version 1.46v67 was then used to quantify
199 immunopositive ZENK cells where the researcher was blind to the groups. The “Analyse
200 Particles” function with in ImageJ was used to count the number of cells within the size range of
201 9.07-27.21 μm^2 , and circularity of 0.40-1.00.

202 3. Results

203 A repeated measures ANOVA using SPSS (IBM SPSS Statistics for Windows, Version
204 22.0 Amronk, NY: IBM Corp.) was conducted with brain region (CMM, NCMd, and NCMv),
205 hemisphere (left vs. right), and section number (1-8) as within-subject factors and playback
206 condition (2 D note *chick-a-dee* calls with low duty cycle, 2 D note *chick-a-dee* calls with high
207 duty cycle, 10 D note *chick-a-dee* calls with high duty cycle, or 2 D note *chick-a-dee* calls with
208 high duty cycle played in reverse) as the between-subject factor. There was a significant main
209 effect of region ($F(2,32) 53.676, p < 0.001$) and hemisphere ($F(1,16) 5.81, p = 0.028$) but no
210 main effect of section number ($F(7,112) 0.581, p = 0.77$), which follows previous auditory
211 ZENK studies (Scully et al., 2017; Avey et al., 2014). We found no significant main effects of
212 playback condition ($F(3,16) 1.199, p = 0.342$; see Figure 3) or significant interaction of playback
213 condition and region ($F(3,16) 0.393, p = 0.760$). Parameter estimates found no significant effect
214 of dependent variables (hemisphere, section number, or brain region) on group when order of fit
215 and effects of independent variables were separately controlled for.

216

217 **4. Discussion**

218 Here we examined the extent to which ZENK expression varied in the auditory brain
219 regions of male chickadees as a function of *chick-a-dee* call composition presented as auditory
220 playback. Specifically, we compared calls with a low or high duty cycle and many or few D
221 notes, to determine whether duty cycle and/or number of D notes presented had an impact on the
222 amount of ZENK expression. We predicted that calls with a high duty cycle would lead to
223 significantly more ZENK expression compared to calls with low duty cycle, whereas calls played
224 in reverse would result in significantly less ZENK expression compared to all other conditions.
225 Contrary to these predictions, we observed similar ZENK expression in response to all playback
226 types, with playback of 2 D low duty cycle and 2 D reversed high duty cycle resulting in ZENK
227 expression not significantly different from 10 D and 2 D high duty cycle stimuli.

228 Overall, our results revealed no statistically significant difference in ZENK expression
229 among any of the groups. Notably, there were no significant differences between high and low
230 duty cycle groups. Regardless of whether birds heard playback with many or few calls per unit
231 time (high vs. low duty cycle), the amount of ZENK expression did not vary significantly. There
232 was also no significant difference between playback of 2 D high duty cycle calls and 10 D high
233 duty cycle calls, suggesting that, neurobiologically at least, both stimuli were treated similarly in
234 terms of the amount of ZENK expression produced. Finally, there was no difference in ZENK
235 expression between the reversed playback control calls and any of the experimental playback
236 groups. This is somewhat surprising since birds respond less behaviorally to reversed call
237 playback (Charrier & Sturdy, 2005), and in some cases also show less ZENK response to
238 reversed call note playback (Avey et al., 2011). The current finding is not unprecedented since in

239 some cases, reversed playback of single notes does not lead to significant reductions in ZENK
240 expression (Scully et al., 2017; Hahn et al., 2015). Our study suggests that reversed playback
241 may not be a compelling control stimulus, particularly in neurobiological studies.

242 **4.1 Comparison with previous work**

243 While we found no difference between our two high duty cycle groups, as we predicted,
244 we also did not find any differences between the low duty cycle group and high duty cycle
245 groups. Because we used the same playback stimuli as Wilson and Mennill (2011), our results
246 suggest that there is an uncoupling between IEG expression and behavior, at least in this case.
247 Birds displayed no significant differences in the amount of ZENK expression whether or not the
248 stimulus would evoke vigorous behavioral responses during field playback studies. Our findings
249 also differ from those of Avey et al. (2011), which reported differences in amount of ZENK
250 expression relative to the number of D notes used in playback stimuli, with calls containing more
251 D notes leading to more ZENK expression. Here, we did not find any difference in ZENK
252 expression between the playback groups with few D notes and many D notes. This may be due to
253 the fact that while our current playback stimuli had many D notes, they were not produced by
254 birds in response to and in the presence of a predator as was the case for the mobbing calls used
255 by Avey et al (2011). The calls used by Avey et al. (2011) may have contained acoustic features
256 or information not present in the edited calls used here and by Wilson and Mennill (2011). In
257 fact, Templeton et al. (2005) reported many fine scale acoustic differences between mobbing
258 calls produced in the presence of high- versus low-threat predators. For example, calls produced
259 in response to high-threat predators had an initial D note with a shorter duration (compared to the
260 other D notes in a call) as well as a shorter interval between the first and second D notes. Calls
261 produced in response to low-threat predators had differences in the spectral structure of D notes

262 compared to D notes produced in response to high-threat predators. Fine scale acoustic features
263 like the ones noted above, were likely present in Avey et al.'s calls and may have led to the
264 observed differences in ZENK expression in Avey et al. (2011). These fine acoustic features are
265 likely not in the calls used in the present study (because of the way in which the calls were
266 constructed) and may underlie our lack of differential ZENK response observed from our
267 different playback conditions.

268 Altering other acoustic features, such as rhythm, has also been studied in songbirds.
269 Zebra finches (*Taeniopygia guttata*) behaviourally differentiate in response to normal and
270 abnormal conspecific songs, and also demonstrate neural differences (Lampen et al., 2017).
271 While rhythm has converging behavioral and neurobiological findings, there is also previous
272 support for our diverging findings. Gobes and colleagues (2009) showed that behaviorally, male
273 zebra finches prefer female calls, but the neural activation in males to female calls did not
274 demonstrate the same trend. While Gobes and colleagues did not alter acoustic features, this is
275 still a strong example of how behavior and neurobiological results do not always line up. It has
276 also been suggested that ZENK is influenced not only by the acoustic properties of the stimuli,
277 but also by attention, arousal, and other environmental factors, which may also need to be further
278 explored (Park & Clayton, 2002). The reasons for the disconnect between ZENK brain response
279 and behavioral response in the field will need to be explored more fully in future work.

280 **4.2 Future directions**

281 We propose several future directions. Most notably, we plan on replicating the current
282 study using the calls used by Avey et al. (2011), but manipulated to vary in duty cycle in a
283 manner consistent with Wilson and Mennill (2011). We will also conduct a study using calls
284 manipulated, following Wilson and Mennill (2011) and this study, but with local calls used as

285 source calls. It might be possible that geographic differences in the calls (collected across North
286 America) were behind the observed differences. We do not think this is likely as previous
287 research has shown that early life experience does not influence neuronal geographic song
288 preference (Hernandez & MacDougall-Shackleton, 2004), but it needs to be ruled out by an
289 experiment designed to test this variable. Finally, replicating Wilson and Mennill's playback
290 study with a local population is also required to ensure that duty cycle is an important feature
291 more generally, and not idiosyncratic of their study population.

292 **4.3 Conclusion**

293 In summary, we showed that differences in *chick-a-dee* call duty cycle, while leading to
294 differential behavioral responses in field playback studies (Wilson & Mennill, 2011), does not
295 lead to differential ZENK immediate early gene expression. Moreover, playback of high duty
296 cycle calls with many D notes does not result in higher levels of ZENK expression than those
297 without many D notes, contrary to previous work by Avey et al. (2011). Resolving these
298 discrepancies and apparent disconnect between behavior and brain will be the focus of future
299 studies.

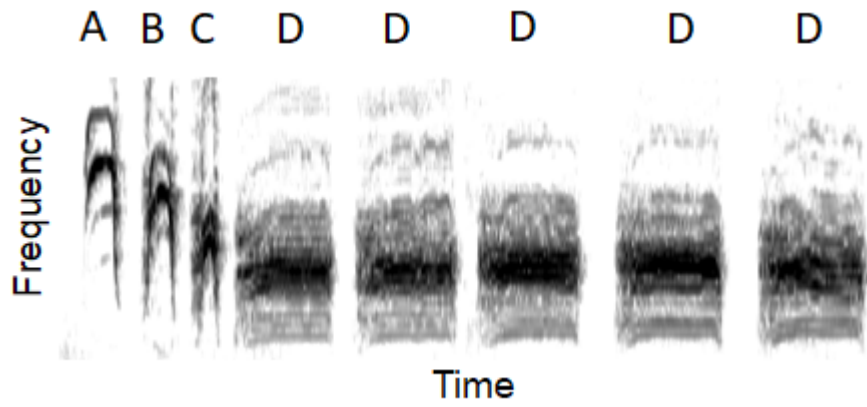
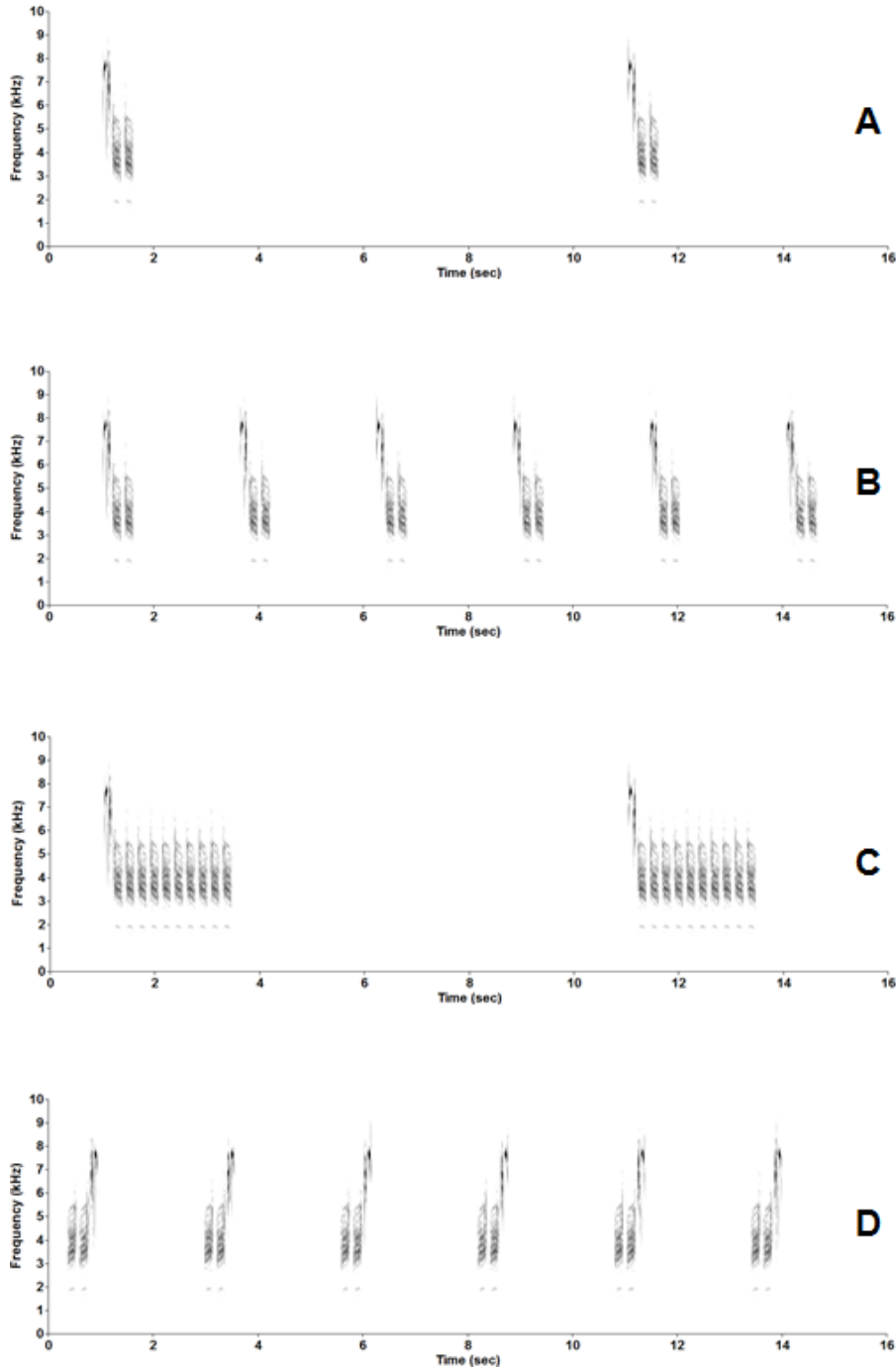


Figure 1: Example of *chick-a-dee* call note types: Spectrogram of a chick-a-dee call demonstrating the four note types: A, B, C, and D.



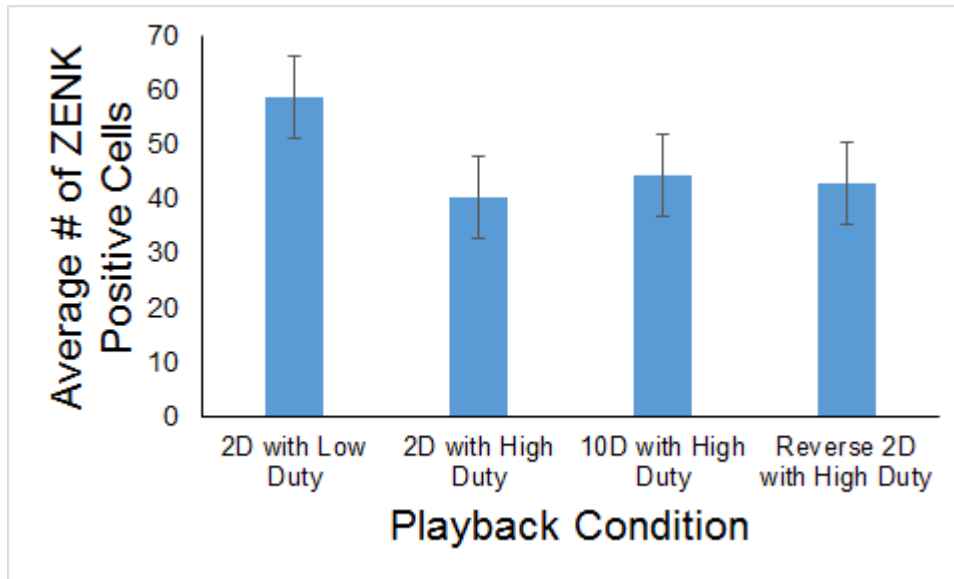
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302 **Figure 2: Spectrograms of Playback Stimuli.** *Chick-a-dee* call with: A) 2 D notes and low duty

303 cycle, B) 2 D notes and high duty cycle, C) 10 D notes and high duty cycle, D) 2 D notes and

304 high duty cycle, but with the call played in reverse.

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308 **Figure 3: Average ZENK expression by playback condition.** A repeated measure ANOVA
309 showed that there was no significant difference between playback conditions, $F(3,16) = 1.199$, p
310 $= 0.342$. The bar graph shows the mean ZENK expression across all areas (standardized across
311 individuals), with error bars representing the SEM.

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