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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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29   **Abstract**

30   Black-capped chickadees (*Poecile atricapillus*) use their namesake *chick-a-dee* call for

31   multiple functions, altering the features of the call depending on context. For example, duty

32   cycle (the proportion of time filled by vocalizations) and fine structure traits (e.g., number of D

33   notes) can encode contextual factors, such as predator size and food quality. Wilson and Mennill

34   (2011) found that chickadees show stronger behavioral responses to playback of *chick-a-dee*

35   calls with higher duty cycles, but not to the number of D notes. That is, independent of the

36   number of D notes in a call, but dependent on the overall proportion of time filled with

37   vocalization, birds responded more to higher duty cycle playback compared to lower duty cycle

38   playback. Here we presented chickadees with *chick-a-dee* calls that contained either two D

39   (referred to hereafter as 2 D) notes with a low duty cycle, 2 D notes with a high duty cycle, 10 D

40   notes with a high duty cycle, or 2 D notes with a high duty cycle but played in reverse (a non-

41   signaling control). We then measured ZENK expression in the auditory nuclei where perceptual

42   discrimination is thought to occur. Based on the behavioral results of Wilson and Mennill (2011),

43   we predicted we would observe the highest ZENK expression in response to forward-playing

44   calls with high duty cycles; we predicted we would observe no significant difference in ZENK

45   expression between forward-playing high duty cycle playbacks (2 D or 10 D). We found no

46   significant difference between forward-playing 2 D and 10 D high duty cycle playbacks.

47   However, contrary to our predictions, we did not find any effects of altering the duty cycle or

48   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,

147   2011 for additional details). Subjects were randomly assigned to one of four groups, with five

148   birds per group, and each group being exposed to one of four types of acoustic stimuli: *chick-a-*

149   *dee* calls with 2 D notes and a low duty cycle, *chick-a-dee* calls with 2 D notes and a high duty

150   cycle, *chick-a-dee* calls with 10 D notes and a high duty cycle, or *chick-a-dee* calls with 2 D

151   notes and a high duty cycle played in reverse. Stimuli consisted of two calls each produced by a

152   different individual. It should be noted that during the *chick-a-dee* calls with 2 D notes and a

153   high duty cycle, there are a greater number of 2-D note calls compared to the number of 10-D

154   note calls during the *chick-a-dee* calls with 10 D notes and a high duty cycle (see Figure 2). In

155   order to avoid pseudoreplication, each bird was presented with different calls (see Kroodsma et

156   al., 2011 for additional details).

157   **2.3 Playback procedure and equipment**

158   Approximately 24 hours before playback, each bird was housed in a cage (Jupiter

159   Parakeet), with access to food and water, in individual soundproof chambers (1.7m x 0.84m x

160   0.58m; Industrial Acoustics Corporation, Bronx, New York, USA) maintained on the natural

161   summer light cycle of Edmonton, Alberta. All birds were exposed to the playback stimulus once

162   a minute, repeated over 30 minutes. After this 30 minutes, birds were exposed to an hour of

163   silence in the dark and then perfused immediately to ensure maximum quantity and quality of

164   ZENK preservation (Mello & Clayton, 1994). A lethal dose of 0.04 ml of 100 mg/ml ketamine

165   and 20 mg/ml xylazine (1:1) was administered intramuscularly to each subject. The bird was

166   perfused via the left ventricle using heparinized 0.1 M phosphate buffered saline (PBS) followed

167   by 4% paraformaldehyde (PFA). The brain of each black-capped chickadee was then extracted

168   and placed in a PFA solution for 24 hours, followed by a 30% sucrose PBS solution for 48 hours.

169   The brains were then fast frozen using isopentane and dry ice and stored at -80°C until sectioned.

2.4 Histology

Brains were sectioned sagittally from the midline, and 48 40μm sections of each hemisphere were collected and stored in PBS. In order to visualize ZENK, sections were first washed twice in 0.1 M PBS for a minimum of five minutes, transferred to a 0.5% H_2O_2 solution and incubated for 15 minutes. Incubation was followed by three 5 min washes in 0.1 M PBS. A second incubation in 10% normal goat serum for 20 hours at room temperature followed. Sections were then transferred into the primary antibody (erg-1, catalogue # sc-189, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 24 hours at a concentration of 1: 5,000 in 0.1 M PBS with Triton X-100 (PSB/T), then washed three times in PBS/T before being incubated in 1:200 biotinylated goat-anti-rabbit antibody (Vector Labs, Burlington, ON, Canada) in PBS/T for one hour. After three more washes in PBS/T, sections were incubated in avidin-biotin horseradish peroxidase (ABC Vectastain Elite Kit; Vector Labs, Burlington, ON, Canada) for one hour, followed by three washes in 0.1M PBS. Sections were then processed with 3,3'-diaminobenzidine tetrachloride (Sigma FastDAB, D4418, Sigma-Aldrich, Santa Fe Springs, CA, USA) to visualize expression of ZENK, followed by three washes with 0.1M PBS to remove any excess visualizing agents.

2.5 Imaging

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

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4. Discussion

Here we examined the extent to which ZENK expression varied in the auditory brain

regions of male chickadees as a function of *chick-a-dee* call composition presented as auditory

playback. Specifically, we compared calls with a low or high duty cycle and many or few D

notes, to determine whether duty cycle and/or number of D notes presented had an impact on the

amount of ZENK expression. We predicted that calls with a high duty cycle would lead to

significantly more ZENK expression compared to calls with low duty cycle, whereas calls played

in reverse would result in significantly less ZENK expression compared to all other conditions.

Contrary to these predictions, we observed similar ZENK expression in response to all playback

types, with playback of 2 D low duty cycle and 2 D reversed high duty cycle resulting in ZENK

expression not significantly different from 10 D and 2 D high duty cycle stimuli.

Overall, our results revealed no statistically significant difference in ZENK expression

among any of the groups. Notably, there were no significant differences between high and low

duty cycle groups. Regardless of whether birds heard playback with many or few calls per unit

time (high vs. low duty cycle), the amount of ZENK expression did not vary significantly. There

was also no significant difference between playback of 2 D high duty cycle calls and 10 D high

duty cycle calls, suggesting that, neurobiologically at least, both stimuli were treated similarly in

terms of the amount of ZENK expression produced. Finally, there was no difference in ZENK

expression between the reversed playback control calls and any of the experimental playback

groups. This is somewhat surprising since birds respond less behaviorally to reversed call

playback (Charrier & Sturdy, 2005), and in some cases also show less ZENK response to

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.
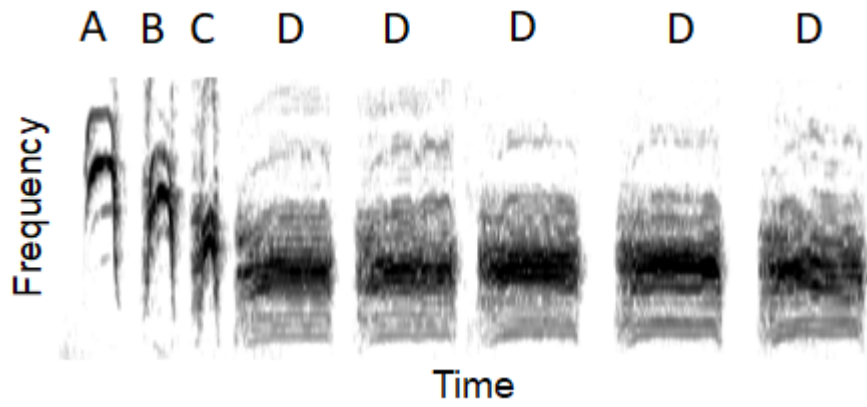
Figure 1: Example of *chick-a-dee* call note types: Spectrogram of a chick-a-dee call demonstrating the four note types: A, B, C, and D.
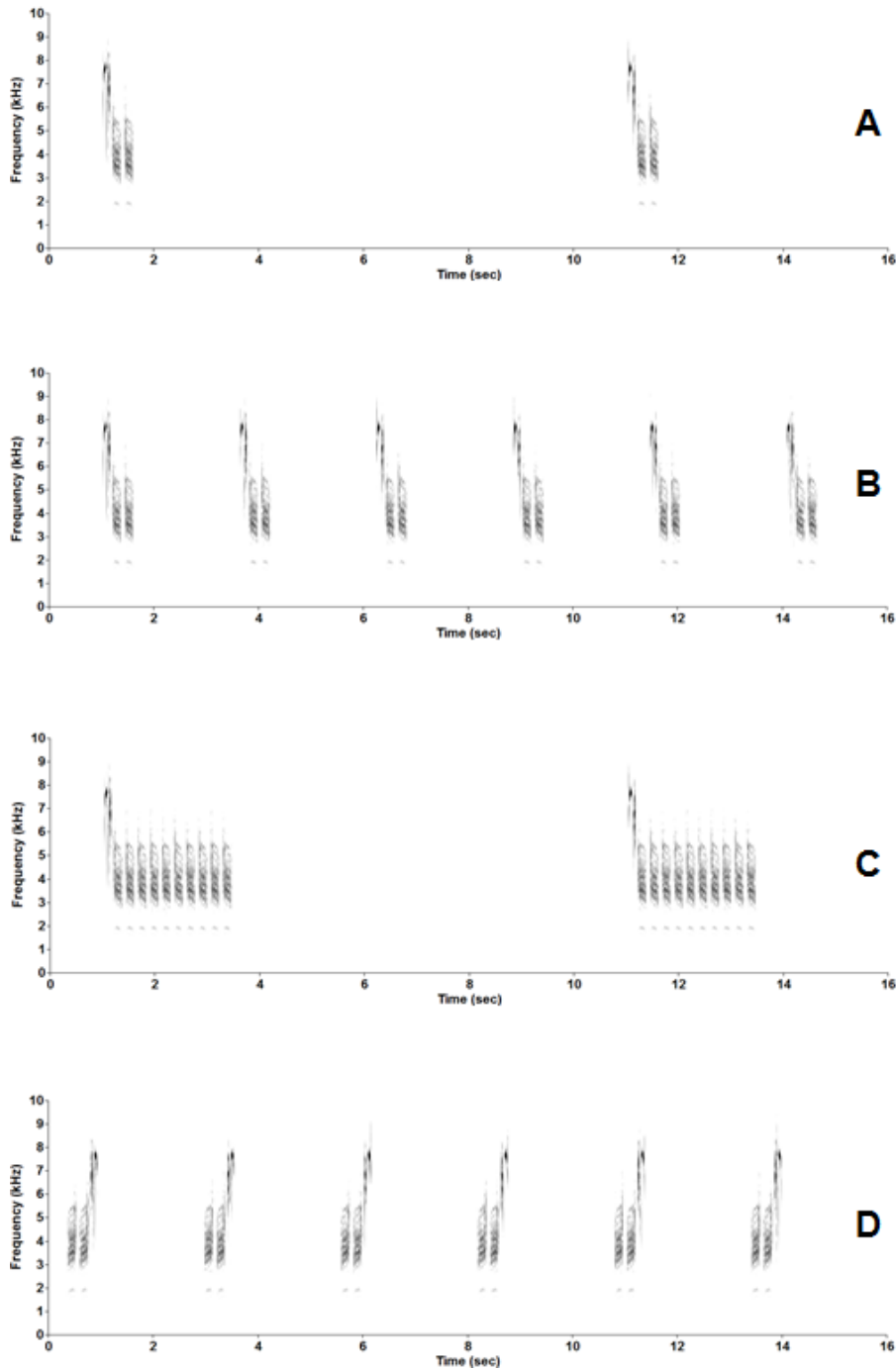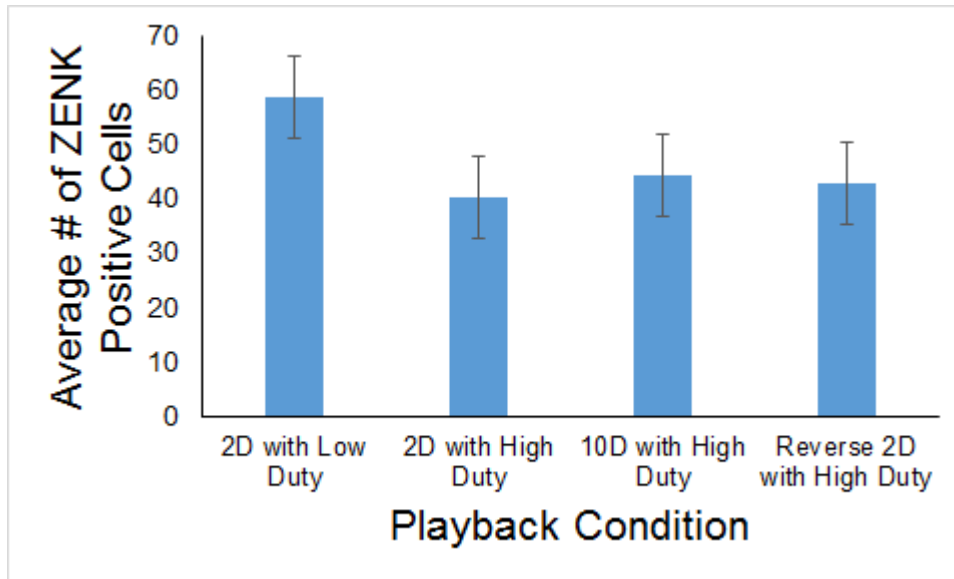
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302   **Figure 2: Spectrograms of Playback Stimuli.** *Chick-a-dee* call with: A) 2 D notes and low duty

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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324

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