

1 **Experience affects immediate early gene expression in response to conspecific call notes in**
2 **black-capped chickadees (*Poecile atricapillus*)**

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14

15 **Abstract**

16 Black-capped chickadees (*Poecile atricapillus*) produce numerous vocalizations, including the
17 acoustically complex *chick-a-dee* call that is composed of A, B, C, and D notes. D notes are
18 longer in duration and lower in frequency than the other note types and contain information
19 regarding flock and species identification. Adult wild-caught black-capped chickadees have been
20 shown to have similar amounts of immediate early gene (IEG) expression following playback of
21 vocalizations with harmonic-like acoustic structure similar to D notes. Here we examined how
22 different environmental experience affects IEG response to conspecific D notes. We hand-reared
23 black-capped chickadees under three conditions: (1) with adult conspecifics, (2) with adult
24 heterospecific mountain chickadees and (3) without adults. We presented all hand-reared birds
25 and a control group of field-reared black-capped chickadees, with conspecific D notes and
26 quantified IEG expression in the caudomedial mesopallium (CMM) and the caudomedial
27 nidopallium (NCM). We found that field-reared birds that heard normal D notes had a similar
28 neural response as a group of field-reared birds that heard playback of reversed D notes. Field-
29 reared birds that heard normal D notes also had a similar neural response as birds reared with
30 adult conspecifics. Birds reared without adults had a significantly reduced IEG response, while
31 the IEG expression in birds reared with heterospecifics was at intermediate levels between birds
32 reared with conspecifics and birds reared without adults. Although acoustic characteristics have
33 been shown to drive IEG expression, our results demonstrate that experience with adults or
34 normal adult vocalizations is also an important factor.

35

36 **Keywords:** black-capped chickadee; songbird; hand-reared; ZENK; auditory perception

37 **1. Introduction**

38 Songbird vocalizations contain species-relevant information that is critical for survival in
39 numerous contexts (e.g., mating, territory defense, flock maintenance) and songbirds are one of a
40 few groups of animals that must have exposure to adult conspecific vocalizations during
41 development in order to produce normal vocalizations as adults (i.e., they are vocal learners;
42 Marler, 1970; 1984; Thorpe, 1958). Both auditory and social experience during ontogeny are
43 important components of normal vocal development (for review see Beecher & Burt, 2004) and
44 influence the processing of auditory signals (e.g., conspecific vocalizations) in the songbird brain
45 (for review see Woolley, 2012). To examine the processing of auditory signals, neuronal
46 activation can be measured by quantifying immediate early gene (IEG) expression in songbird
47 auditory forebrain areas (e.g., caudomedial mesopallium, CMM; caudomedial nidopallium,
48 NCM). Early studies quantifying the expression of the IEG ZENK (*zif-268*, *egr-1*, NGFI-A,
49 *krox-24*) in adult songbirds demonstrated more neuronal activation following playback of
50 conspecific vocalizations compared to heterospecific vocalizations, tones, or silence (e.g., Mello
51 et al., 1992; Mello & Clayton, 1994).

52 Recently, Avey et al. (2014) found that playback of vocalizations with similar acoustic
53 structure led to similar amounts of neuronal activation in CMM and NCM of black-capped
54 chickadees, suggesting that neuronal activity is also influenced by the acoustic features of the
55 signal. Avey et al. (2014), however, examined wild-caught adult chickadees, so all birds had
56 experience with normal conspecific vocalizations. The current study examines the influence that
57 experience with adult vocalizations has on the neuronal activation in the auditory forebrain areas
58 (specifically, CMM and NCM) in black-capped chickadees.

59 Most studies examining the effects of early experience focus on the perception of male
60 songs (but see Vicario et al., 2002); however, songbird calls are also a biologically important
61 vocalization used for maintaining flock cohesion, indicating a food source, raising alarm and
62 mobbing predators (Marler, 2004). The *chick-a-dee* call produced by black-capped chickadees is
63 a learned vocalization (Hughes et al., 1998) comprised of four note-types produced in a relatively
64 fixed order: A → B → C → D; however, the number of each note-type within a call can vary
65 each time it is produced (Ficken et al., 1978). Compared to the other note-types within the call, D
66 notes are lower in frequency and longer in duration. D notes contain species-specific information
67 (Bloomfield & Sturdy 2008; Bloomfield et al., 2008a; Guillette et al., 2010), and all species in
68 the genus *Poecile* produce a variant of the *chick-a-dee* call. D notes also have multiple
69 biologically important functions (e.g., flock identification: black-capped chickadees, Mammen &
70 Nowicki, 1981; recruiting flock mates to a food source: Carolina chickadees, *Poecile*
71 *carolinensis*, Mahurin & Freeberg, 2009; information regarding predator threat level: black-
72 capped chickadees, Templeton et al., 2005; tufted titmice, *Baeolophus bicolor*, Courter &
73 Ritchison, 2010; Carolina chickadees, Soard & Ritchison, 2009). Because D notes are a salient
74 component of the *chick-a-dee* call, we examined the effects of experience on ZENK expression
75 in adult black-capped chickadees following playback of conspecific D notes.

76 We reared black-capped chickadees under three conditions: (1) with conspecific adult
77 black-capped chickadees, (2) with heterospecific adult mountain chickadees, and (3) without
78 adults. Birds hand-reared in the laboratory were housed under these conditions through
79 adulthood until testing in the current experiment (see Material and methods for details). During
80 the experiment, birds hand-reared under the three conditions were presented with playback of
81 conspecific black-capped chickadee D notes. We also presented field-reared black-capped

82 chickadees playback of either: (1) conspecific D notes, or (2) conspecific D notes played in
83 reverse. Following playback we quantified the amount of ZENK expression in auditory forebrain
84 areas. We had four main predictions: (1) field-reared adults would have lower ZENK expression
85 following playback of reversed D notes compared to field-reared birds exposed to normal D
86 notes, (2) birds hand-reared in the presence of adult conspecifics would have similar amounts of
87 ZENK expression to field-reared adult black-capped chickadees, (3) birds hand-reared with a
88 closely-related heterospecific chickadee species (i.e., mountain chickadees) would have similar
89 amounts of ZENK expression compared to birds hand-reared with adult conspecifics, and (4)
90 birds reared in the absence of adults will have significantly less ZENK expression compared to
91 birds hand-reared with adult conspecifics or adult heterospecifics.

92 Our first prediction is based on the results of Avey et al. (2014) which found significantly
93 more ZENK expression following playback of normal black-capped chickadee D notes
94 compared to reversed D notes. Examining these two conditions also allowed us to compare
95 ZENK expression in field-reared birds following playback of vocalizations the birds had
96 previous experience with to ZENK expression in field-reared birds following playback of
97 vocalizations the birds did not have previous experience with (i.e., reversed D notes), while still
98 controlling for overall spectral and temporal complexity of the playback vocalizations. For our
99 remaining three predictions, we compared birds that heard the same playback types, but birds
100 had different environmental experiences from early rearing through adulthood. Field-reared
101 adults and birds hand-reared with adult conspecifics both had experience with conspecific D
102 notes, but birds in these two groups differed in the rearing environment (i.e., field versus
103 laboratory). In mountain chickadees, captivity reduces hippocampal volume but not the number
104 of hippocampal neurons (LaDage et al., 2009) and similar results have been found for black-

105 capped chickadees, with environmental experience affecting hippocampal volume, but not
106 hippocampal neuron number (Roth, et al., 2012). This suggests that while neuron number (at
107 least in the hippocampus) is relatively stable, lab housing does affect neuronal architecture. In
108 the current study, by comparing field-reared adults and birds hand-reared with adult conspecifics,
109 we could determine if captivity had an effect on the number of immediate early genes that were
110 expressed in auditory areas.

111 We also compared birds hand-reared with black-capped chickadees to birds hand-reared
112 with mountain chickadees; while birds in these two groups were all hand-reared in the
113 laboratory, they differed in the acoustic environment they were exposed to. We predicted no
114 difference between birds reared in these two conditions, because, while black-capped and
115 mountain chickadees produce acoustically distinct D notes, the notes also have acoustic
116 similarities (e.g., complex harmonic-like structure) and hand-reared birds in both of these rearing
117 conditions had experience with adult chickadee vocalizations. Previous studies have found no
118 difference in discrimination abilities for black-capped chickadees reared under these two
119 conditions (Bloomfield et al., 2008b), suggesting similar auditory processing between these
120 groups. In addition, black-capped chickadees hearing playback of vocalizations with similar
121 acoustic structure as D notes results in the same amount of ZENK expression compared to birds
122 hearing playback of conspecific D notes, suggesting that previous experience with a vocalization
123 acoustically similar to the playback vocalization is enough to elicit similar levels of ZENK
124 expression (Avey et al., 2014). Finally, we compared birds hand-reared without adults to birds
125 hand-reared with adults because birds in these two groups differed in their early acoustic
126 experience, with the former group having no experience with adults during development. We
127 predicted less ZENK expression in birds reared without adults based on studies with other

128 songbirds, which have demonstrated that experience with adult models and adult vocalizations
129 affects auditory processing (e.g., Cousillas et al., 2004; Cousillas et al., 2006; George et al.,
130 2010).

131

132 **2. Material and methods**

133 *2.1 Subjects*

134 Eleven black-capped chickadees were collected from three nest sites around Edmonton,
135 AB, Canada (53.48°N, 113.55°W; 53.47°N, 113.56°W; 53.36°N, 112.89°W) between 10-14 days
136 post hatch in June 2008. Birds were hand-reared until independence (approximately 30-35 days
137 old), and then were housed individually under one of three conditions: (1) in a colony room with
138 conspecific adult black-capped chickadees (hereafter referred to as BCCH-reared); (2) in a
139 colony room with heterospecific adult mountain chickadees (referred to as MOCH-reared); or (3)
140 in a sound attenuating chamber with no adult birds, but in the presence of the other hand-reared
141 birds (referred to as ISO-reared). Birds were housed under these conditions from 30-35 days post
142 hatch until being sacrificed in the current experiment.

143 Eight black-capped chickadees were wild-caught as adults (at least one year of age,
144 determined by the color and shape of the outer tail retrices, Pyle, 1997) between January 2009
145 and March 2012 in and around Edmonton, AB, Canada (North Saskatchewan River Valley,
146 53.53°N, 113.53°W; Mill Creek Ravine, 53.52°N, 113.47°W; Stony Plain, 53.46°N, 114.01°W)
147 and were housed in colony rooms with conspecifics (referred to as field-reared). All housing
148 rooms were maintained on a light:dark cycle that mimicked the natural light cycle for Edmonton,
149 AB, Canada. Sex was initially determined by DNA analysis (Griffiths et al., 1998) and was
150 confirmed by post-mortem identification of the gonads. See Guillette et al. (2011) for details

151 regarding the housing and care for hatchling and adult birds. All experimental procedures were
152 approved by the Animal Care and Use Committee for Biosciences at the University of Alberta
153 and were carried out in accordance with guidelines from the Canadian Council on Animal Care.

154

155 *2.2 Recordings and acoustic analysis*

156 To better understand the auditory experience of each group of hand-reared chickadees,
157 we recorded the hand-reared birds approximately one year after capture. We also recorded wild-
158 caught adult black-capped and mountain chickadees (three individuals of each species). An
159 individual bird was placed in a sound-attenuating chamber ($1.7 \times 0.8 \times 0.58$ m; Industrial
160 Acoustics Corporation, Bronx, NY) and recorded using an AKG C 1000S (AKG Acoustics,
161 Vienna, Austria) microphone connected to a Marantz PMD670 (Marantz America, Mahwah, NJ)
162 digital recorder (16 bit; 44,100 Hz sampling rate).

163 Our objective for the vocal analysis was to determine if birds in the three hand-reared
164 groups were producing, and in turn hearing, D notes and how similar the acoustic structure of
165 these D notes was to the acoustic structure of species-typical adult black-capped chickadee D
166 notes (i.e., the notes used as stimuli in the current experiment). Specifically, we were interested
167 in the acoustic structure of D notes produced and heard by birds in the ISO-reared group,
168 because the acoustic experience of these birds was limited to the vocalizations produced by the
169 individuals in the rearing condition.

170 From each bird, we randomly selected 20 *chick-a-dee* call D notes to measure. One
171 female in the BCCH-reared group only produced one D note so we excluded this bird from the
172 analysis. To standardize our measurements, each D note was saved as a separate file with a
173 duration of 500 ms by adding silence to the beginning and end of each sound file. We made four

174 acoustic measurements: total duration, frequency of the first visible harmonic, loudest frequency,
175 and note peak frequency (i.e., loudest frequency in the highest harmonic when additional
176 harmonics occur). The temporal measurement was made using a sound spectrogram with a
177 spectrogram window size of 256 points and time resolution 5.8 ms. We used a cutoff amplitude
178 of -35 to 0 dB relative to note peak amplitude. The three frequency measurements were made
179 using a power spectrum with a window size of 32,768 points and frequency resolution of 1.3 Hz
180 (88 Hz smoothing). These measurements have been used previously in the analyses of *chick-a-*
181 *dee* calls by adult black-capped and mountain chickadees (Charrier et al. 2004; Bloomfield et al.
182 2004).

183 We conducted a stepwise discriminant function analysis using SPSS (version 21.0.0.0,
184 SPSS, Inc, Chicago, IL) to classify call notes in terms of which individual produced the
185 vocalization. For this analysis, we used the leave-one-out method of cross-validation. With this
186 method, one vocalization is withheld and a discriminant function is derived from the remaining
187 cases. Using the derived discriminant function, the withheld case is then classified and this
188 process is repeated until all vocalizations have been classified (Betz, 1987). From the predictions
189 of the discriminant function analysis we evaluated the classification errors to examine which
190 individual the discriminant function analysis would classify a call as being produced by when it
191 was not classified as being produced by the correct individual. By examining the prediction
192 errors, we could gain an understanding of subtle differences in the D notes produced by birds
193 with different environmental experiences. More specifically, we could determine if D notes
194 produced by black-capped chickadees that were housed in different environments (i.e., with adult
195 conspecifics, with adult heterospecifics, or without adults) would be misclassified as being
196 produced by field-reared black-capped chickadees, suggesting that they are acoustically similar

197 to normal D notes, and thus similar to the D notes used as acoustic stimuli in the experiment (see
198 Playback stimuli, below).

199

200 *2.3 Playback stimuli*

201 Black-capped chickadee calls were recorded in the field at Elk Island National Park, AB,
202 Canada (53°36N, 112°51W) with a Marantz PMD670 digital recorder (16 bit, 44,100 Hz
203 sampling rate) and a Sennheiser ME67 (Saul Mineroff Electronics, Elmont, NY, USA; frequency
204 response range 40-20,000 Hz) directional microphone. Calls were bandpass filtered between 500
205 Hz and 14,000 Hz using Goldwave (Goldwave, St. John's, NL, Canada) to remove background
206 noise and amplitude was equalized using SIGNAL 5.0 sound analysis software (Engineering
207 Design, Berkeley, CA, USA).

208 We created two types of stimulus sets: black-capped chickadee *chick-a-dee* call D notes,
209 and reversed black-capped chickadee *chick-a-dee* call D notes (Figure 1). Four field-reared adult
210 chickadees (2 males; 2 females) heard the forward D notes and four field-reared adult chickadees
211 (2 males; 2 females) heard the reversed D notes. All hand-reared birds heard the forward D
212 notes. Details regarding the construction of the stimulus sets can be found in Avey et al. (2014).
213 In short, there were four stimulus sets, and each bird within a rearing condition heard a different
214 stimulus set. Each stimulus set contained four D notes produced by two black-capped
215 chickadees. These four notes were played within a 10 s period followed by 50 s of silence, to
216 make up one min of playback. This one minute was repeated 30 times for a total playback time
217 of 30 min.

218

219 *2.4 Playback equipment*

220 Stimuli were played through either a Cambridge Azur 351A Integrated Amplifier or
221 Cambridge Azur 640A Integrated Amplifier (Cambridge Audio, London, England; frequency
222 response range 5-50,000 Hz) and a Fostex FE108E Σ full-range speaker (Fostex Corp., Japan;
223 frequency response range 80-18,000 Hz) from an mp3 player (Creative ZEN; Singapore). Stimuli
224 were played at approximately 74 dB as measured by a Brüel & Kjær Type 2239A integrating
225 sound level meter (A weighting, slow response; Brüel & Kjær Sound & Vibration Measurement
226 A/S, Nærum, Denmark). Playback was conducted in sound-attenuating chambers (1.7 m \times 0.84
227 m \times 0.58 m; Industrial Acoustics Company, Bronx, NY).

228

229 *2.5 Playback Procedure*

230 Playback was conducted between 22 April and 2 May 2013. Individual chickadees were
231 housed in acoustic chambers overnight in a modified home cage, containing three perches, two
232 water bottles, and two food cups, providing *ad libitum* access to water and food. Birds were
233 monitored and recorded before stimulus playback during 30 minutes of silence and during 30
234 minutes of playback using an AKG C 1000S microphone connected to a Marantz PMD670
235 digital recorder and a Sony Handycam DCR-SX45 (Sony Corporation of America, NY, USA).
236 Following playback, the lights were extinguished for 1 h.

237

238 *2.6 Histology*

239 Following the 1 h of darkness, birds were immediately given an overdose (approximately
240 0.03 ml) of 100 mg/ml ketamine and 20 mg/ml xylazine (1:1) delivered intramuscularly. Birds
241 were transcardially perfused with heparinized 0.1M phosphate buffered saline (PBS) followed by
242 4% paraformaldehyde. The brain was extracted and placed in 4% paraformaldehyde for at least

243 24 hours and then put in a 30% sucrose PBS solution at 4°C until fully saturated (approximately
244 24 hours). Brains were frozen with isopentane and stored at -80°C until immunocytochemistry
245 (ICC) for ZENK protein was conducted.

246 Forty-eight 40µm sagittal sections were collected using a cryostat from each brain
247 hemisphere starting at the midline and proceeding laterally. Sections were placed in 0.1M PBS
248 and we processed brains in batches randomized across treatment groups. Sections were washed
249 twice for at least five minutes in 0.1M PBS, incubated in 0.5% H₂O₂ in distilled H₂O for 15 min,
250 washed three more times for five minutes per wash in 0.1M PBS, and incubated in 10% Normal
251 Goat Serum (catalogue # S-1000, Vector Laboratories, Burlington, ON, Canada) for 20 hours.
252 Sections were then incubated in a 1:5000 concentration of primary antibody (egr-1, catalogue #
253 sc-189, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 0.1M PBS containing Triton X-100
254 (PBS/T) for 24 hours. Sections were washed three times for five minutes per wash in PBS/T and
255 incubated in biotinylated goat anti-rabbit antibody (catalogue # BA-1000, Vector Laboratories)
256 for 1 h (1:250 dilution in PBS/T), washed again three times for five minutes per wash in PBS/T
257 and incubated in avidin-biotin horseradish peroxidase complex (Vectastain Elite ABC Kit,
258 Vector Laboratories) for 1 h (1:200 dilution in PBS/T). Next, sections were washed three times
259 in PBS/T (five minutes per wash) and visualized with 3,3'-diaminobenzidine tetrachloride
260 (Sigma FastDAB, D4418; Oakville, ON, Canada). Finally, sections were mounted on gelatin-
261 coated microscope slides, dehydrated in a series of ethanol concentrations, cleaned with Citrisolv
262 (Fisher Scientific, Ottawa, ON, Canada) and protected with cover slips using Permount (Sigma-
263 Aldrich, Oakville, ON, Canada).

264

265 *2.7 Analysis*

266 We quantified ZENK expression in CMM and the dorsal and ventral section of NCM
267 (NCMd and NCMv, respectively). The lateral ventricle and the caudal-ventral boundary of the
268 mesopallial lamina (LaM) defined the most caudal area of CMM where ZENK expression was
269 quantified. The lateral ventricle defined the dorsal, ventral, and caudal borders of NCM and the
270 rostral border was defined by Field L, which is an area with very little ZENK expression. Similar
271 methods have been used previously to quantify ZENK expression in CMM and NCM (Avey et
272 al. 2008a; 2011a). Sixteen sections (eight per hemisphere) were measured for ZENK expression.
273 We began quantifying ZENK expression on the first section in which the mesopallium was
274 contiguous with the rostral portion of the nidopallium to make sure orientation of the
275 nidopallium was correct. We took three images (0.20 mm × 0.15 mm) from each of the 16 brain
276 sections (one image per auditory region), so in total, 48 images per bird were captured using a
277 Leica microscope (DM5500B; Wetzlar, Germany) with a 40× objective and a Retiga Exi camera
278 (Qimaging, Surrey, BC, Canada) using Openlab 5.1 (Perkin Elmer Inc, Waltham, MA, USA).
279 Figure 2 shows example ZENK expression from each condition.

280 Immunoreactive cells were counted using ImageJ (1.47v, NIH, Bethesda, MD, USA).
281 Images were first converted to greyscale, auto-contrasted, and the auto threshold algorithm was
282 used to identify immunoreactive nuclei from surrounding tissue. Cells that ranged from $9.07\mu\text{m}^2$
283 to $27.21\mu\text{m}^2$ were counted. We conducted manual counts on a subset of images ($n = 78$), and
284 found the counts generated using ImageJ and the manual counts were highly correlated ($r = .88$,
285 $p \leq 0.001$). During imaging and cell counting the experimenter was blind to the bird's rearing
286 and playback condition.

287

288 3. Results

289 We conducted a repeated measures analysis of variance (ANOVA) using Statistica 12
290 (StatSoft, Inc, Tulsa, OK) with brain region (CMM, NCMd, NCMv), hemisphere (left, right),
291 and medial-lateral position (1-8) as within-subject factors and rearing condition as a between-
292 subjects factor. This analysis revealed a significant main effect for rearing condition ($F_{4,14} =$
293 $3.31, p = 0.042$; field-reared adult (forward D note), $M = 222.90$; BCCH-reared, $M = 184.28$;
294 MOCH-reared, $M = 115.92$; ISO-reared, $M = 56.42$; field-reared adult (reversed D note), $M =$
295 196.28 ; see Figure 3). There was a significant main effect of brain region (Wilks' λ ; $F_{2,13} =$
296 $24.62, p < 0.001$; CMM, $M = 165.00$; NCMd, $M = 162.26$; NCMv, $M = 133.63$). There was no
297 significant main effect of hemisphere (Wilks' λ ; $F_{1,14} = 1.84, p = 0.20$) or medial-lateral position
298 (Wilks' λ ; $F_{7,8} = 2.78, p = 0.09$). There was a significant rearing condition \times medial-lateral
299 position interaction (Wilks' λ ; $F_{28,30} = 1.87, p = 0.048$); there were no other significant
300 interactions.

301 We conducted a post hoc analysis (Tukey's HSD) to examine the significant main effect
302 of brain region and found that there was significantly more expression in CMM and NCMd
303 compared to NCMv (both $ps < 0.001$), but expression in CMM and NCMd was not significantly
304 different ($p = 0.87$; Figure 4). To examine our *a priori* predictions for differences between birds
305 in the different rearing conditions, we conducted planned comparisons. There were no significant
306 differences in expression for BCCH-reared chickadees compared to field-reared birds ($p = 0.51$)
307 or MOCH-reared birds ($p = 0.25$). ISO-reared birds had significantly less expression compared
308 to BCCH-reared birds ($p = 0.04$), but not compared to MOCH-reared birds ($p = 0.28$). Field-
309 reared birds hearing D notes in reverse had on average less expression compared to field-reared
310 birds hearing forward D notes (see Figure 3), but this difference was not statistically significant
311 ($p = 0.62$).

312

313 3.1 Vocal analysis

314 Table 1 shows the mean \pm standard deviation of the four acoustic features that we
315 measured. We conducted a discriminant function analysis to classify call notes based on the
316 individual that produced the vocalization. The overall Wilks' λ was significant [Wilks' $\lambda = 0.07$,
317 $\chi^2(60, N = 320) = 824, p < 0.001$], indicating that overall, the model used the predictor variables
318 to discriminate among the individuals. The squared canonical correlation coefficient (R_c^2)
319 indicates how much of the variation is explained by the predictor variables in the discriminant
320 function. Four discriminant functions (Function 1 eigenvalue = 2.94, $R_c^2 = 0.75$; Function 2
321 eigenvalue = 1.42, $R_c^2 = 0.59$; Function 3 eigenvalue = 0.37, $R_c^2 = 0.27$; Function 4 eigenvalue =
322 0.10, $R_c^2 = 0.09$) assigned 40.0% of the cross-validated cases to the correct individual (chance =
323 $1/16 = 6.25\%$). Table 2 shows the number of calls produced by each individual that were
324 correctly classified by the discriminant function analysis and the number of calls that were
325 misclassified as being produced by other individuals, and includes the rearing condition for each
326 individual. Interestingly, for field-reared mountain chickadees, most of the call notes were either
327 correctly classified, or misclassified as being produced by other field-reared mountain
328 chickadees (85%). For field-reared black-capped chickadees, most of the call notes were either
329 correctly classified, or misclassified as being produced by field-reared black-capped chickadees
330 (60%) or call notes were misclassified as being produced by BCCH-reared individuals (28.3%).
331 For BCCH-reared birds, most call notes were correctly classified or misclassified as being
332 produced by other BCCH-reared birds (55%) or were misclassified as being produced by field-
333 reared black-capped chickadees (25%). These results suggest that the notes are acoustically
334 similar to notes produced by conspecific birds with the same environmental experience (e.g.,

335 black-capped chickadees hand-reared with conspecifics were acoustically similar to other
336 BCCH-reared birds or adult field-reared conspecifics). For MOCH-reared and ISO-reared birds,
337 very few call notes were misclassified as being produced by adult field-reared black-capped
338 chickadees (7.5% and 0%, respectively), suggesting that the vocalizations produced (and, in the
339 case of the ISO-reared birds, the vocalizations heard) are not acoustically similar to D notes
340 produced by adult field-reared black-capped chickadees (i.e., normal D notes).

341

342 **4. Discussion**

343 Our study demonstrates that in black-capped chickadees environmental experience
344 affects the amount of ZENK expression in the auditory forebrain areas CMM and NCM,
345 suggesting that the acoustic properties of signals from the environment during early development
346 and onwards influence the auditory processing of vocalizations in adults. In the current study, we
347 predicted that reversed D notes would result in lower ZENK expression compared to forward D
348 notes in field-reared birds, but we found no difference in ZENK expression for field-reared birds
349 hearing these two stimulus types. In agreement with our predictions, we found that birds reared
350 in the presence of conspecific black-capped chickadees had similar amounts of ZENK expression
351 compared to field-reared birds, and that black-capped chickadees reared with heterospecific
352 mountain chickadees had similar amounts of ZENK expression compared to birds reared with
353 conspecifics. Also in line with our predictions, we found that birds reared in isolation from adults
354 had significantly less ZENK expression compared to birds reared with conspecifics; however, in
355 contrast to our predictions, birds reared with heterospecifics had similar amounts of ZENK
356 expression compared to birds reared in isolation from adults.

357

358 4.1 Expression in field-reared adults

359 We predicted that field-reared chickadees hearing forward D notes would have more
360 ZENK expression compared to field-reared adults hearing reversed call notes, based on the
361 results of Avey et al. (2014) that showed significantly more ZENK expression following
362 playback for forward D notes compared to reversed D notes. However, in the current study,
363 although there was less ZENK expression for birds that heard playback of reversed D notes the
364 difference was not statistically significant. In Avey et al.'s study, the absolute amount of ZENK
365 expression was still high for birds that heard playback of reversed D notes. Avey et al.'s study
366 was conducted in December when *chick-a-dee* calling is high, while the current experiment was
367 conducted at the end of April, when *chick-a-dee* call production is low (Avey et al. 2008b;
368 2011b), and it is possible that seasonal differences played a role in the different results of the two
369 studies. Further work is needed to clarify if season affects IEG expression to forward or reversed
370 D notes. A previous study with black-capped chickadees did not find seasonal differences in
371 ZENK expression in birds that heard whole *chick-a-dee* calls or *fee-bee* songs, but found
372 seasonal differences in expression in birds that heard heterospecific (song sparrow) songs
373 (Phillmore et al. 2011). When birds hear full *chick-a-dee* calls comprised of reversed notes,
374 chickadees respond with fewer *chick-a-dee* calls compared to playback of normal calls (Charrier
375 & Sturdy, 2005), but birds' abilities at discriminating individual forward and reversed call notes
376 based on our results here and previous work of Avey et al. (2014) appears to be less sensitive and
377 therefore requires further examination. While there are fine acoustic differences in a note played
378 forward compared to reversed (e.g., onset and offset frequencies), the current results suggest that
379 the overall spectral and temporal complexity of the notes influenced the ZENK expression.

380 In the current experiment, no birds had prior experience with D notes played in reverse,
381 but all field-reared birds had prior experience with forward D notes, and the similarities in
382 acoustic complexity that exist between forward and reversed D notes may explain the similar
383 levels of ZENK expression for these two playback conditions. More research is needed to
384 examine the extent to which acoustic complexity of a signal and auditory experience influence
385 the auditory processing of signals. If ZENK expression was being driven by both prior auditory
386 experience and the acoustic complexity of the signal, we would expect to find similar amounts of
387 IEG expression if we played reversed D notes to birds reared with conspecifics or
388 heterospecifics, while we would expect less expression in birds reared in isolation.

389

390 *4.2 Effects of rearing with conspecific or heterospecific adults*

391 We found that for black-capped chickadees reared in the presence of adult conspecifics
392 there was no difference in the amount of ZENK expression compared to field-reared birds. This
393 is evidence that our lab-rearing did not affect the auditory processing of vocalizations when birds
394 had vocal and visual contact with adults.

395 For birds that were reared with adult mountain chickadees (heterospecifics) we found
396 similar levels of ZENK expression compared to birds reared with conspecifics, suggesting that
397 having experience with adult conspecifics was not the critical factor in producing the genomic
398 responses. This result is in agreement with the behavioral results from Bloomfield et al. (2008b)
399 suggesting that early experience with either chickadee species is sufficient for perceptual abilities
400 similar to field-reared birds.

401 Phylogenetically, mountain chickadees are the closest relative to black-capped
402 chickadees (Gill et al., 2005) and mountain chickadees also produce D notes within their *chick-a-*

403 *dee* call. In captive housing, our chickadees produce species-typical vocalizations that vary
404 seasonally in a manner similar to that of wild chickadees (Avey et al. 2008b; 2011b), so birds
405 reared with either mountain or black-capped chickadees heard species-typical (i.e., black-capped
406 or mountain chickadee) vocalizations, including *chick-a-dee* call D notes. Although black-
407 capped and mountain chickadee D notes contain acoustically distinct, discriminable features
408 (Dawson et al., 2006) and evidence suggests that D notes contain species-specific information
409 (Bloomfield et al., 2008a; Guillette et al., 2010), D notes produced by black-capped and
410 mountain chickadees also contain acoustic similarities (e.g., fundamental frequency and loudest
411 frequency, for review see Guillette et al., 2013). The acoustic similarities between the two
412 species' call notes may be responsible for the high level of ZENK expression found for the
413 MOCH-reared birds (i.e., expression was not different from the BCCH-reared group), even
414 though the playback stimuli (i.e., conspecific D notes) were vocalizations with which the birds
415 had no prior experience. This interpretation is consistent with the results of Avey et al. (2014),
416 where acoustic similarity, not phylogenetic relationship, drove ZENK expression in these brain
417 areas. The current results for the MOCH-reared group suggests that the acoustic complexity of
418 the playback call note, and not experience with the call note, was driving the expression, but we
419 cannot rule out the possibility that experience with an acoustically similar vocalization (i.e.,
420 experience with heterospecific D notes) was also a critical factor. The level of ZENK expression
421 in birds in the MOCH-reared group was not statistically different from the level of ZENK
422 expression in birds reared without adults, indicating that the neuronal activation for birds in the
423 MOCH-reared group was intermediate between the BCCH-reared and ISO-reared groups. This
424 suggests that not having exposure to conspecific D notes may have also hindered the neural

425 response of birds in the MOCH-reared group (i.e., level of expression was similar to birds reared
426 without adults).

427

428 *4.3 Effects of rearing without adults*

429 Birds reared without adults had significantly lower ZENK expression compared to birds
430 reared in the presence of adult conspecifics. The birds reared in isolation from adults were kept
431 under these conditions from day 10-14 post-hatch until the time of the current experiment
432 (approximately five years). We do not know the critical length of adult isolation that was
433 responsible for our findings, or if a shorter duration would have led to different results. In other
434 species, recent auditory and/or social experience in adult songbirds can alter the response in
435 auditory forebrain areas (Sockman et al., 2002; Terleph et al., 2008) and IEG expression is
436 increased when birds are in the presence of conspecifics (Vignal et al., 2005), demonstrating the
437 importance of social context to neuronal activation. In starlings, isolation from adults during the
438 period of song acquisition leads to deficits in auditory neural responses, even if birds hear
439 conspecific songs during the first few months post-hatch (George et al., 2010). In canaries
440 (*Serinus canaria*) that are socially isolated early in development, later socialization can change
441 some aspects of singing behavior and brain anatomy to match birds that were never isolated
442 (Leitner & Catchpole, 2007).

443 In the current experiment, birds were reared without adults but were not raised in total
444 isolation, and they had opportunities to hear other hand-reared conspecific vocalizations;
445 however, the results from the discriminant function analysis suggest that the vocalizations
446 produced by birds reared without adults were acoustically dissimilar to D notes produced by
447 field-reared birds. The results from the other playback conditions suggest that overall auditory

448 experience (specifically, the acoustic features within the vocalizations that are heard) or adult
449 presence is critical to neuronal activation in the auditory areas we examined.

450

451 *4.4 Effects of rearing on vocal production*

452 Birds in each hand-reared condition were producing (and therefore hearing) D notes (see
453 Figure 5 for an example of D notes produced by birds in each hand-rearing condition). Results
454 from the discriminant function analysis suggest that the D notes produced by birds in the ISO-
455 reared group were not acoustically similar to field-reared black-capped chickadee D notes. It is
456 possible that the lower ZENK expression in ISO-reared birds compared to BCCH-reared birds
457 was because ISO-reared birds did not have previous exposure to species-typical D notes.

458 Similarly, the results from the discriminant function analysis suggest that D notes produced by
459 birds in the MOCH-reared group were not acoustically similar to field-reared black-capped
460 chickadees, indicating that birds reared under this condition also did not have experience with
461 species-typical conspecific vocalizations (i.e., birds in this condition were not reared with adult
462 conspecifics). However, the ZENK expression in birds in the MOCH-reared group was
463 intermediate between BCCH-reared and ISO-reared, suggesting that prior experience with
464 species-typical conspecific D notes was not the only factor driving the ZENK expression. These
465 results indicate that social context (i.e., experience with adults or adult vocalizations) is also an
466 important factor in auditory perception and influences neuronal activation. To our knowledge,
467 the role that auditory experience plays in the development of D notes within the *chick-a-dee* call
468 has not been examined previously.

469 Baker et al. (2003) examined black-capped chickadee vocal development in the field, and
470 found juveniles began producing D notes before producing A, B, or C notes. The study by Baker

471 et al. (2003) found that by post-hatch day 10, some birds were producing D notes with some
472 similarity to adult D notes, and chickadees were producing typical D notes by post-hatch day 32.
473 In the current study, birds were removed from the nest 10-14 days post hatch, so it is possible
474 that birds were already beginning to produce D-like notes before they were brought into the
475 laboratory. The results from our discriminant function analysis suggests that birds reared without
476 adult conspecifics (i.e., MOCH- and ISO-reared) were not producing species-typical notes.
477 However, additional studies are needed to further examine the development of D notes when
478 chickadees are isolated from adults compared to birds reared in the presence of adults. Previous
479 research has shown that black-capped chickadees learn B and C notes within their call, while
480 species-typical A notes are not dependent on early experience with adults; however, D note
481 production was not examined (Hughes et al., 1998).

482

483 *4.5 Conclusion*

484 Black-capped chickadees have a complex communication system, comprised of
485 numerous vocalizations, including the acoustically complex *chick-a-dee* call. This study adds to
486 a growing body of literature examining the importance of auditory experience in developing
487 perceptual abilities in black-capped chickadees. In the current study we found that experience
488 with either conspecific or closely-related heterospecific species resulted in similar levels of IEG
489 expression following playback of a conspecific vocalization; similarly, Bloomfield et al. (2008b)
490 found that chickadees reared with conspecifics or heterospecifics had no rearing-specific
491 advantage in a discrimination task. We found that birds reared without adults had less IEG
492 expression compared to birds reared with conspecifics, but not compared to birds reared with
493 heterospecifics. This suggests that experience with conspecific adults (or adult vocalizations) is

494 important for neuronal activation, but experience with closely-related heterospecific adults (or
495 adult vocalizations) can also lead to increased neuronal activation. Black-capped chickadees
496 reared in the absence of adults have been shown to perceive distance cues similar to field-reared
497 birds, but hand-reared birds have deficits in discriminating individual vocalizations (Phillmore et
498 al., 2003b) and relative pitch (Njegovan and Weisman, 1997). To our knowledge, the current
499 study is the first to examine ZENK expression in a group of black-capped chickadees that was
500 never exposed to adult vocalizations (but see Phillmore et al. 2003a; Avey et al. 2011a for
501 studies that examined IEG response in hand-reared chickadees with some experience with adult
502 vocalizations).

503

504 **Acknowledgements**

505 All animal studies were conducted in accordance with the Canadian Council on Animal Care
506 Guidelines and Policies and with approval from the Animal Care and Use Committee for
507 Biosciences for the University of Alberta, and the University of Calgary Life and Environmental
508 Sciences Animal Care Committee. Chickadees were captured and research was carried out under
509 an Environment Canada Canadian Wildlife Service Scientific permit, Alberta Fish and Wildlife
510 Capture and Research permits, and City of Edmonton Parks Permit. This research was supported
511 by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant
512 and Discovery Accelerator Supplement, an Alberta Ingenuity Fund (AIF) New Faculty Grant, a
513 Canada Foundation for Innovation (CFI) New Opportunities Fund (NOF) and Infrastructure
514 Operating Fund (IOF) grants along with start-up funding and CFI partner funding from the
515 University of Alberta (UofA) to CBS. LMG was supported by an Izaak Walton Killam Memorial

516 Scholarship (IWKMS) at UofA, and is currently supported by a Newton International Fellowship
517 jointly run by the Royal Society and the British Academy.

518

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655

656 **Figure Captions**

657 Figure 1. Sound spectrograms of (A) black-capped chickadee D note and (B) reversed black-
658 capped chickadee D note (transform length = 256 points; -35 to 0 dB relative to peak amplitude).

659

660 Figure 2. Example ZENK expression in CMM, NCMd, and NCMv for black-capped chickadees
661 in each condition: field-reared (forward D notes); black-capped chickadee-reared (BCCH-
662 reared); mountain chickadee-reared (MOCH-reared); isolate-reared (ISO-reared); and field-
663 reared (reversed D notes). Scale bar = 50 μ m.

664

665 Figure 3. Mean number of ZENK positive cells for black-capped chickadees in each condition.
666 Error bars represent standard error of the mean.

667

668 Figure 4. Mean number of ZENK positive cells for each brain region (CMM, NCMd, NCMv).
669 Averaged across playback conditions. Error bars represent standard error of the mean.

670

671 Figure 5. Sound spectrograms (transform length = 256 points; -35 to 0 dB relative to peak
672 amplitude) of D notes produced by birds in each rearing condition. Notes produced by (A,B)
673 black-capped chickadee-reared, (C,D) mountain chickadee-reared, and (E,F) isolate-reared birds.
674 Each note was produced by a different individual.

675

676 **Tables**

677

678 Table 1. Mean \pm standard deviation of the four acoustic features measured in D notes produced
 679 by mountain chickadees wild-caught as adults (MOCH field-reared), black-capped chickadees
 680 wild-caught as adults (BCCH field-reared), black-capped chickadees hand-reared with black-
 681 capped chickadees (BCCH-reared), black-capped chickadees hand-reared with mountain
 682 chickadees (MOCH-reared), and black-capped chickadees hand-reared without adults (ISO-
 683 reared). TD = total duration of the note; F0 = frequency of first visible harmonic; Fmax = loudest
 684 frequency; NPF = note peak frequency.

| Group | TD | F0 | Fmax | NPF |
|------------------------|--------------------|----------------------|----------------------|-----------------------|
| MOCH (field-reared) | 261.76 \pm 56.44 | 1991.82 \pm 232.30 | 4206.60 \pm 270.02 | 7960.13 \pm 74064 |
| BCCH (field-reared) | 200.98 \pm 14.38 | 1538.58 \pm 108.88 | 3428.27 \pm 205.64 | 7235.59 \pm 662.64 |
| BCCH- reared | 221.98 \pm 14.38 | 1515.45 \pm 143.04 | 3377.15 \pm 320.23 | 7948.55 \pm 980.97 |
| MOCH- reared | 296.35 \pm 55.47 | 1665.96 \pm 146.55 | 3378.68 \pm 241.52 | 6514.93 \pm 1015.17 |
| ISO-reared | 280.47 \pm 44.61 | 1590.89 \pm 148.74 | 3385.33 \pm 353.13 | 6937.40 \pm 1064.91 |

685

686 Table 2. Matrix of classification by individual using the leave-one-out method of cross-
687 validation (n = 20 call notes per individual). Number of call notes correctly classified as
688 produced by the correct individual are presented along the diagonal and indicated with an
689 asterisk (*). Misclassifications are presented in corresponding rows and columns under the
690 predicted individual's identification. Overall, 40% of cross-validated cases were correctly
691 classified. Individual birds are organized based on which rearing group they belong to: MOCH
692 field-reared = mountain chickadees wild-caught as adults, BCCH field-reared = black-capped
693 chickadees wild-caught as adults, BCCH-reared = black-capped chickadee hand-reared with
694 black-capped chickadees, MOCH-reared = black-capped chickadees hand-reared with mountain
695 chickadees, ISO-reared = black-capped chickadees hand-reared without adults. Values in bold
696 are the number and percent (in parentheses) of call notes classified as produced by an individual
697 in each rearing group. Values bolded in italics are call notes that are classified as produced by the
698 correct individual or an individual within the same rearing group as the actual individual who
699 produced the call.

| | Predicted | | | | | | | | | | | | | | | | | | | |
|----------------------|---------------------|-------|-------|-------|---------------------|-------|-------|-------|--------------------|-----|-----|-----|--------------------|-----|-----|-----|--------------------|-----|-----|--|
| | MOCH (field-reared) | | | | BCCH (field-reared) | | | | BCCH-reared | | | | MOCH-reared | | | | ISO-reared | | | |
| | 3478 | 3483 | 3486 | | 48110 | 48118 | 48123 | | LYB | RBG | LWB | RGR | RRG | RWG | RWR | RZR | LBG | LBZ | | |
| Actual | 9* | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 3 | 0 | 0 | | | |
| MOCH (field-reared) | 3478 | 3483 | 3486 | 3478 | 3483 | 3486 | 48110 | 48118 | 48123 | LYB | RBG | LWB | RGR | RRG | RWG | RWR | RZR | LBG | LBZ | |
| | 9* | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 3 | 0 | 0 | 0 | 0 | |
| | 3 | 9* | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | |
| | 0 | 7 | 13* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| Total notes (n=60) | 51 (85%) | | | | 2 (3.33%) | | | | 0 (0%) | | | | 4 (6.67%) | | | | 3 (5%) | | | |
| BCCH (field-reared) | 48110 | 48118 | 48123 | 48110 | 48118 | 48123 | LYB | RBG | LWB | RGR | RRG | RWG | RWR | RZR | LBG | LBZ | | | | |
| | 0 | 0 | 0 | 5* | 5 | 2 | 0 | 5 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| | 1 | 0 | 0 | 2 | 6* | 4 | 1 | 4 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | |
| | 0 | 0 | 0 | 1 | 0 | 11* | 0 | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| Total notes (n = 60) | 1 (1.67%) | | | | 36 (60%) | | | | 17 (28.33%) | | | | 5 (8.33%) | | | | 1 (1.67%) | | | |
| BCCH-reared | LYB | RBG | LWB | RGR | RRG | RWG | RWR | RZR | LBG | LBZ | | | | | | | | | | |
| | 0 | 0 | 0 | 0 | 1 | 0 | 11* | 2 | 3 | 2 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | |
| | 1 | 0 | 0 | 0 | 4 | 5 | 3 | 6* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | |
| Total notes (n = 40) | 1 (2.5%) | | | | 10 (25%) | | | | 22 (55%) | | | | 6 (15%) | | | | 1 (2.5%) | | | |
| MOCH-reared | LWB | RGR | RRG | RWG | RWR | RZR | LBG | LBZ | | | | | | | | | | | | |
| | 0 | 0 | 0 | 0 | 1 | 1 | 4 | 1 | 2 | 3* | 1 | 1 | 0 | 2 | 1 | 1 | 1 | 2 | 0 | |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 13* | 0 | 1 | 1 | 0 | 4 | 0 | 0 | 0 | |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 8* | 2 | 1 | 4 | 2 | 0 | 0 | 0 | |
| | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 2 | 12* | 0 | 2 | 2 | 0 | 0 | 0 | |
| Total notes (n = 80) | 1 (1.25%) | | | | 6 (7.5%) | | | | 4 (5%) | | | | 47 (58.75%) | | | | 22 (27.5%) | | | |
| ISO-reared | RWR | RZR | LBG | LBZ | | | | | | | | | | | | | | | | |
| | 1 | 0 | 0 | 0 | 5 | 0 | 0 | 1 | 7* | 3 | 0 | 0 | 1 | 2 | 2* | 0 | 2 | 0 | 1 | |
| | 0 | 2 | 0 | 0 | 1 | 2 | 1 | 3 | 2 | 5 | 1 | 1 | 3 | 0 | 0 | 7* | 0 | 0 | 2 | |
| | 1 | 0 | 0 | 0 | 2 | 0 | 0 | 2 | 1 | 5 | 1 | 3 | 0 | 0 | 0 | 0 | 7* | 0 | 0 | |
| | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 2 | 4 | 0 | 0 | 0 | 1 | 2 | 0 | 0 | 6* | 0 | |
| Total notes (n = 80) | 4 (5%) | | | | 0 (0%) | | | | 14 (17.5%) | | | | 29 (36.25%) | | | | 33 (41.25%) | | | |