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Research report

ZENK expression in the auditory pathway of black-capped chickadees (*Poecile atricapillus*) as a 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.

1. Introduction

Songbirds possess a unique vocal organ, the syrinx, that allows them to communicate with individuals of both their own and other species using vocalizations of varying complexity [2]. Changes in the structural patterns of these vocalizations are easily noticeable by songbirds, and do not need to be taught [3]. *Chick-a-dee* calls, produced by multiple Paridae species, including black-capped chickadees (*Poecile atricapillus*), are used to convey a variety of information, such as threat posed by predators [4], recruitment to food sources [5], recruitment of conspecifics and heterospecifics to mob a perched predator [6], as well as species-specific information [7]. Chickadees are a popular model species used for exploring the mechanisms behind information coding in acoustic signals, due to the complexity and relative sophistication of *chick-a-dee* calls (see [1]).

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

Previous research has examined the vocal and behavioral responses of chickadees hearing *chick-a-dee* calls of varying acoustic structure. For example, Templeton et al. [4] demonstrated that, in general, black-capped chickadees produce mobbing calls containing more D notes in

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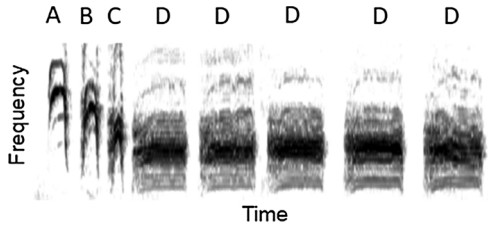

Fig. 1. Example of *chick-a-dee* call note types: Spectrogram of a chick-a-dee call demonstrating the four note types: A, B, C, and D.

response to the presence of smaller, more agile, high-threat predators (compared to larger, less agile, low-threat predators). This suggests that number of D notes conveys the degree of threat posed by predators. In contrast, Wilson and Mennill [1] demonstrated that the duty cycle (i.e., the proportion of time that a call can be heard) of *chick-a-dee* calls, not the signal structure (e.g., note composition in the call), dictates the level of behavioral response by conspecifics to playback of *chick-a-dee* calls; playback with high duty cycles attracted more conspecific receivers, elicited quicker and closer approaches, and responding birds remained within 10 m of the playback speaker for longer than playback with low duty cycle. Furthermore, they found that a receiver's behavioral response did not differ as a function of the number of D notes; responses to both high duty cycle playback of calls with few D notes and high duty cycle playback of calls with many D notes were statistically indistinguishable, suggesting that duty cycle, not the number of D notes, is the salient feature (see [1]).

While variations in call properties have been demonstrated to elicit differential behavioral responses such as the number of conspecific receivers attracted, as well as the rate of approach by receivers [1], changes in call properties have also been found to lead to differential amounts of immediate early gene (IEG) expression in Parid auditory areas. These varied neural responses signify neural plasticity and altered perception in response to a changing auditory environment. For example, it has been shown that *chick-a-dee* mobbing calls in response to high threat predators have a corresponding higher expression of the IEG Zif268/Egr-1/NGFI-A/Krox-24 (ZENK) in telencephalic auditory areas [i.e., caudomedial mesopallium (CMM) and caudomedial nidopallium (NCM); see [8]]. Therefore, expression of IEG such as ZENK in the auditory areas may provide insight into how receivers perceive differences in duty cycle and call structure.

In the current study, we examined the amount of ZENK expression in the telencephalic auditory areas of black-capped chickadees prompted by auditory playback of variations of *chick-a-dee* calls, specifically variation in fine structure (i.e., number of D notes) and sequence-level parameters (i.e., duty cycle). Based on previous neurobiological [8] and behavioral results [1] our primary aim was to explore the independent and combined effects of variation in call structure and variation in duty cycle on IEG expression. Using male chickadees, we conducted a playback experiment with four conditions varying in both duty cycle and number of D notes (Fig. 2): (1) *chick-a-dee* calls containing 2 D notes with a low duty cycle, (2) *chick-a-dee* calls containing 2 D notes with a high duty cycle, (3) *chick-a-dee* calls containing 10 D notes with a high duty cycle, and (4) *chick-a-dee* calls containing 2 D notes with a high duty cycle but played in reverse, thereby creating a non-biologically-relevant stimulus and serving as a negative control (as in [8]). The duty cycle was identical between the 2 D note and 10 D note high duty cycle groups, so any differences in IEG expression would be due to perceptual differences in response to the number of D notes. Similarly, the 2 D note high duty cycle and low duty cycle groups had identical call structure, so any differences would be due to perceptual differences in response to duty cycle.

Based on Wilson and Mennill's [1] results, we predicted that the highest levels of ZENK expression would be found following playback of *chick-a-dee* calls with high duty cycles; specifically, we predicted that *chick-a-dee* calls containing 2 D notes with a high duty cycle and *chick-a-dee* calls containing 10 D notes with a high duty cycle would elicit similar levels of ZENK expression.

2. Methods

2.1. Subjects

Twenty male black-capped chickadees caught from three sites in Edmonton, Alberta, Canada (North Saskatchewan River Valley, 53.53 N, 113.53 W; Mill Creek Ravine, 53.52 N, 113.47 W; Stony Plain, 53.46 N, 114.01 W) were used in this study. All birds were captured between 24 December 2010 and 26 January 2013, and were at least one year of age when captured (identified by examining the color and shape of the rectrices [9,10];). Post-capture, birds were housed indoors in individual Jupiter Parakeet cages (30 × 40 × 40 cm, Rolf C. Hagen Inc, Montreal, QB, Canada) that enabled visual and auditory, but not physical, contact with other male and female black-capped chickadees. Colony rooms were kept on the natural light cycle of Edmonton, and maintained at 20 degrees Celsius. Subjects were given *ad libitum* access to food (Mazuri Small Bird Maintenance Diet; Mazuri, St. Louis, MO, U.S.A), water, grit, cuttlebone, and various environmental enrichment materials (perches, separators, houses). A mixture of egg and spinach or parsley, worms, and water supplements (Prime Vitamin Supplement; Hagen, Inc.) were given on alternating days.

2.2. Playback stimuli

Our playback stimuli were a subset of the *chick-a-dee* calls with varying duty cycles and/or number of D notes that were originally constructed and used by Wilson and Mennill [1]. Briefly, calls were obtained from a variety of sources, produced by several individual chickadees, and were edited to create playback stimuli that were either low duty cycle with 2 D notes or high duty cycle with either 2 D or 10 D notes. The 2 D high duty cycle stimuli and the 10 D note high duty cycle stimuli had identical duty cycles, to test the effect of fine structure (i.e., number of D notes) rather than duty cycle. Calls were modified to contain a certain number of notes, but each call contained notes produced by a single individual (see [1] 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 Fig. 2). In order to avoid pseudoreplication, each bird was presented with different calls (see [11] for additional details).

2.3. Playback procedure and equipment

Approximately 24 h before playback, each bird was housed in a cage (Jupiter Parakeet), with access to food and water, in individual soundproof chambers (1.7 m x 0.84 m x 0.58 m; 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 min. After this 30 min, birds were exposed to an hour of silence in the dark and then perfused immediately to ensure maximum quantity and quality of ZENK preservation [12]. 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%

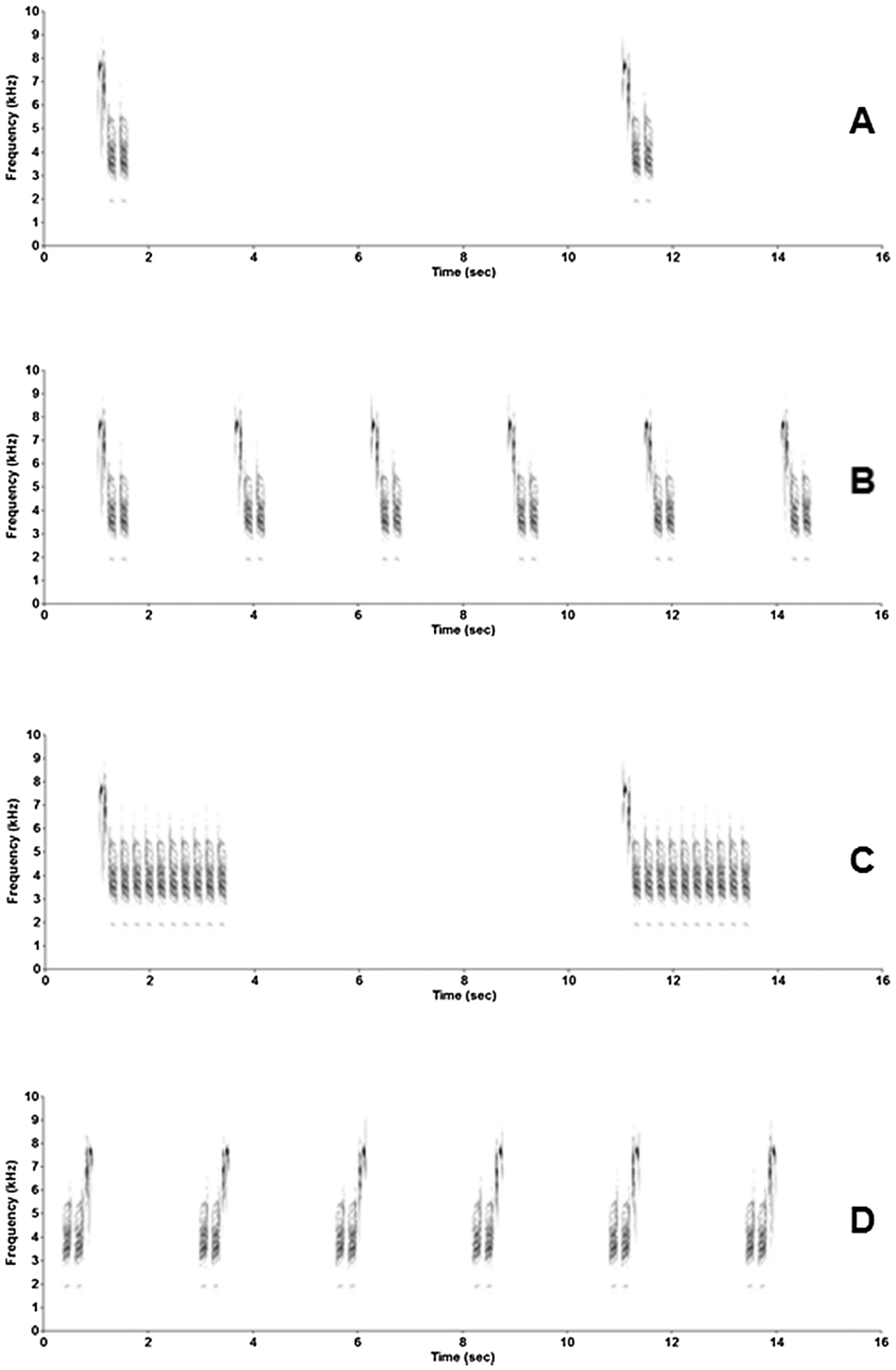

Fig. 2. Spectrograms of Playback Stimuli. *Chick-a-dee* call with: A) 2 D notes and low duty cycle, B) 2 D notes and high duty cycle, C) 10 D notes and high duty cycle, D) 2 D notes and high duty cycle, but with the call played in reverse.

paraformaldehyde (PFA). The brain of each black-capped chickadee was then extracted and placed in a PFA solution for 24 h, followed by a 30% sucrose PBS solution for 48 h. 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 40 μm 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 min. Incubation was followed by three 5 min washes in 0.1 M PBS. A second incubation in 10% normal goat serum for 20 h 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 h at a concentration of 1: 5000 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 0.1 M 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 0.1 M 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 using a 40x objective lens, a Retiga Exi camera (Qimaging, Surrey, BC, Canada), and Openlab 5.1 on a Macintosh OS X (Version 10.4.11). To ensure that each area was imaged in the same location across slices and brains, we captured one image at each location as described in Avey et al. [13]. Overlap in the ventral and dorsal regions of the NCM was carefully avoided by imaging the ventral-most and dorsal-most portions as there are no distinguishing landmarks between the two areas [14]. ImageJ version 1.46v67 was then used to quantify immunopositive ZENK cells where the researcher was blind to the groups. The “Analyse Particles” function with in ImageJ was used to count the number of cells within the size range of 9.07–27.21? ?m^2, and circularity of 0.40–1.00.

3. Results

A repeated measures ANOVA using SPSS (IBM SPSS Statistics for Windows, Version 22.0 Amronk, NY: IBM Corp.) was conducted with brain region (CMM, NCMd, and NCMv), hemisphere (left vs. right), and section number (1–8) as within-subject factors and playback condition (2 D note *chick-a-dee* calls with low duty cycle, 2 D note *chick-a-dee* calls with high duty cycle, 10 D note *chick-a-dee* calls with high duty cycle, or 2 D note *chick-a-dee* calls with high duty cycle played in reverse) as the between-subject factor. There was a significant main effect of region (F(2,32) 53.676, $p < 0.001$) and hemisphere (F(1,16) 5.81, $p = 0.028$) but no main effect of section number (F(7,112) 0.581, $p = 0.77$), which follows previous auditory ZENK studies [14,15]. We found no significant main effects of playback condition (F(3,16) 1.199, $p = 0.342$; see Fig. 3) or significant interaction of playback condition and region (F(3,16) 0.393, $p = 0.760$). Parameter estimates found no significant effect of dependent variables (hemisphere, section number, or brain region) on group when order of fit and effects of independent variables were separately controlled for.

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.

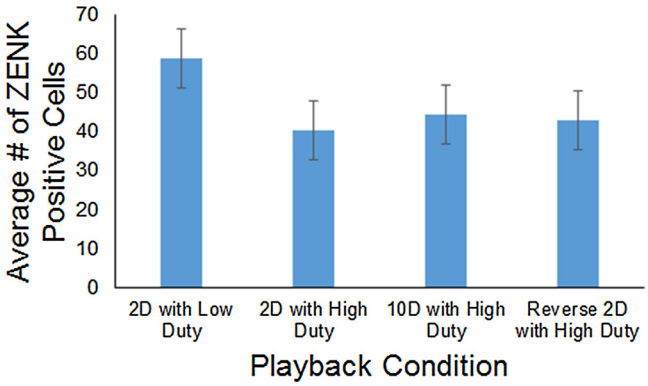

Fig. 3. Average ZENK expression by playback condition. A repeated measure ANOVA showed that there was no significant difference between playback conditions, F(316) = 1.199, $p = 0.342$. The bar graph shows the mean ZENK expression across all areas (standardized across individuals), with error bars representing the SEM.

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 [7], and in some cases also show less ZENK response to reversed call note playback [8]. The current finding is not unprecedented since in some cases, reversed playback of single notes does not lead to significant reductions in ZENK expression [15,16]. Our study suggests that reversed playback may not be a compelling control stimulus, particularly in neurobiological studies.

4.1. Comparison with previous work

While we found no difference between our two high duty cycle groups, as we predicted, we also did not find any differences between the low duty cycle group and high duty cycle groups. Because we used the same playback stimuli as Wilson and Mennill [1], our results suggest that there is an uncoupling between IEG expression and behavior, at least in this case. Birds displayed no significant differences in the amount of ZENK expression whether or not the stimulus would evoke vigorous behavioral responses during field playback studies. Our findings also differ from those of Avey et al. [8], which reported differences in amount of ZENK expression relative to the number of D notes used in playback stimuli, with calls containing more D notes leading to more ZENK expression. Here, we did not find any difference in ZENK expression between the playback groups with few D notes and many D notes. This may be due to the fact that while our current playback stimuli had many D notes, they were not produced by birds in response to and in the presence of a predator as was the case for the mobbing calls used by Avey et al [8]. The calls used by Avey et al. [8] may have contained acoustic features or information not present in the edited calls used here and by Wilson and Mennill [1]. In fact, Templeton et al. [4] reported many fine scale acoustic differences between mobbing calls produced in the presence of high- versus low-threat predators. For example, calls produced in response to high-threat predators had an initial D note with a shorter duration (compared to the other D notes in a call) as well as a shorter interval between the first and second D notes.

Calls produced in response to low-threat predators had differences in the spectral structure of D notes compared to D notes produced in response to high-threat predators. Fine scale acoustic features like the ones noted above, were likely present in Avey et al.'s calls and may have led to the observed differences in ZENK expression in Avey et al. [8]. These fine acoustic features are likely not in the calls used in the present study (because of the way in which the calls were constructed) and may underlie our lack of differential ZENK response observed from our different playback conditions.

Altering other acoustic features, such as rhythm, has also been studied in songbirds. Zebra finches (*Taeniopygia guttata*) behaviourally differentiate in response to normal and abnormal conspecific songs, and also demonstrate neural differences [17]. While rhythm has converging behavioral and neurobiological findings, there is also previous support for our diverging findings. Gobes et al. [18] showed that behaviorally, male zebra finches prefer female calls, but the neural activation in males to female calls did not demonstrate the same trend. While Gobes and colleagues did not alter acoustic features, this is still a strong example of how behavior and neurobiological results do not always line up. It has also been suggested that ZENK is influenced not only by the acoustic properties of the stimuli, but also by attention, arousal, and other environmental factors, which may also need to be further explored [19]. The reasons for the disconnect between ZENK brain response and behavioral response in the field will need to be explored more fully in future work.

4.2. Future directions

We propose several future directions. Most notably, we plan on replicating the current study using the calls used by Avey et al. [8], but manipulated to vary in duty cycle in a manner consistent with Wilson and Mennill [1]. We will also conduct a study using calls manipulated, following Wilson and Mennill [1] and this study, but with local calls used as source calls. It might be possible that geographic differences in the calls (collected across North America) were behind the observed differences. We do not think this is likely as previous research has shown that early life experience does not influence neuronal geographic song preference [20], but it needs to be ruled out by an experiment designed to test this variable. Finally, replicating Wilson and Mennill's playback study with a local population is also required to ensure that duty cycle is an important feature more generally, and not idiosyncratic of their study population.

5. Conclusion

In summary, we showed that differences in *chick-a-dee* call duty cycle, while leading to differential behavioral responses in field playback studies [1], does not lead to differential ZENK immediate early gene expression. Moreover, playback of high duty cycle calls with many D notes does not result in higher levels of ZENK expression than those without many D notes, contrary to previous work by Avey et al. [8]. Resolving these discrepancies and apparent disconnect between behavior and brain will be the focus of future studies.

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