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Research report

ZENK expression following conspecific and heterospecific playback in the zebra finch auditory forebrain

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ABSTRACT

Zebra finches (*Taeniopygia guttata*) are sexually dimorphic songbirds, not only in appearance but also in vocal production: while males produce both calls and songs, females only produce calls. This dimorphism provides a means to contrast the auditory perception of vocalizations produced by songbird species of varying degrees of relatedness in a dimorphic species to that of a monomorphic species, species in which both males and females produce calls and songs (e.g., black-capped chickadees, *Poecile atricapillus*). In the current study, we examined neuronal expression after playback of acoustically similar hetero- and conspecific calls produced by species of differing phylogenetic relatedness to our subject species, zebra finch. We measured the immediate early gene (IEG) ZENK in two auditory areas of the forebrain (caudomedial mesopallium, CMM, and caudomedial nidopallium, NCM). We found no significant differences in ZENK expression in either male or female zebra finches regardless of playback condition. We also discuss comparisons between our results and the results of a previous study conducted by Avey et al. [1] on black-capped chickadees that used similar stimulus types. These results are consistent with the previous study which also found no significant differences in expression following playback of calls produced by various heterospecific species and conspecifics [1]. Our results suggest that, similar to black-capped chickadees, IEG expression in zebra finch CMM and NCM is tied to the acoustic similarity of vocalizations and not the phylogenetic relatedness of the species producing the vocalizations.

1. Introduction

Songbirds produce and respond to vocal communication signals for many biologically-important functions. For example, many species produce vocalizations to attract a potential mate and defend their territory from rivals. In these cases, it is important for animals to be able to distinguish among individuals within their own species, but it is also important for animals to recognize species identity (e.g., to ensure mating with a conspecific). While many previous studies have looked at behavioral responses to heterospecific vocalizations, there has been relatively little research on neural responses in recent years. Here we use zebra finches, a commonly studied vocal learning species, to examine neural responses following playback of heterospecific and conspecific vocalizations.

Zebra finches are sexually dimorphic, with only males producing song. During development, males learn their song from tutors, whereas females develop song preferences by listening to male tutors [2]. Although only males sing, both male and female zebra finches produce distance calls, the main call of the zebra finch, used for identity, alarm, and localization. Distance calls are made up of two components: a tonal portion that contains complex harmonics and ends with a higher frequency than the start of the note; and a noise (broadband) portion that is characterized by a downward harmonic sweep, giving this portion of the call a more harsh sound (Fig. 1a & b; [2]. In male calls, the two components are easily distinguishable, starting with a higher frequency portion that then decreases rapidly in frequency, ending with a longer, more harmonic portion of slightly lower frequency (Fig. 1b). Each call may contain differing numbers of tonal and noise notes in any order, although one tonal note followed by one noise note is the most common sequence. While both male and female zebra finches produce this call, as shown in Figs. 1a and 1b, calls produced by each sex differ in composition and duration, with female calls typically being longer and less acoustically complex than male calls [2]. In comparison, the female call has one main frequency band, which is similar to the second

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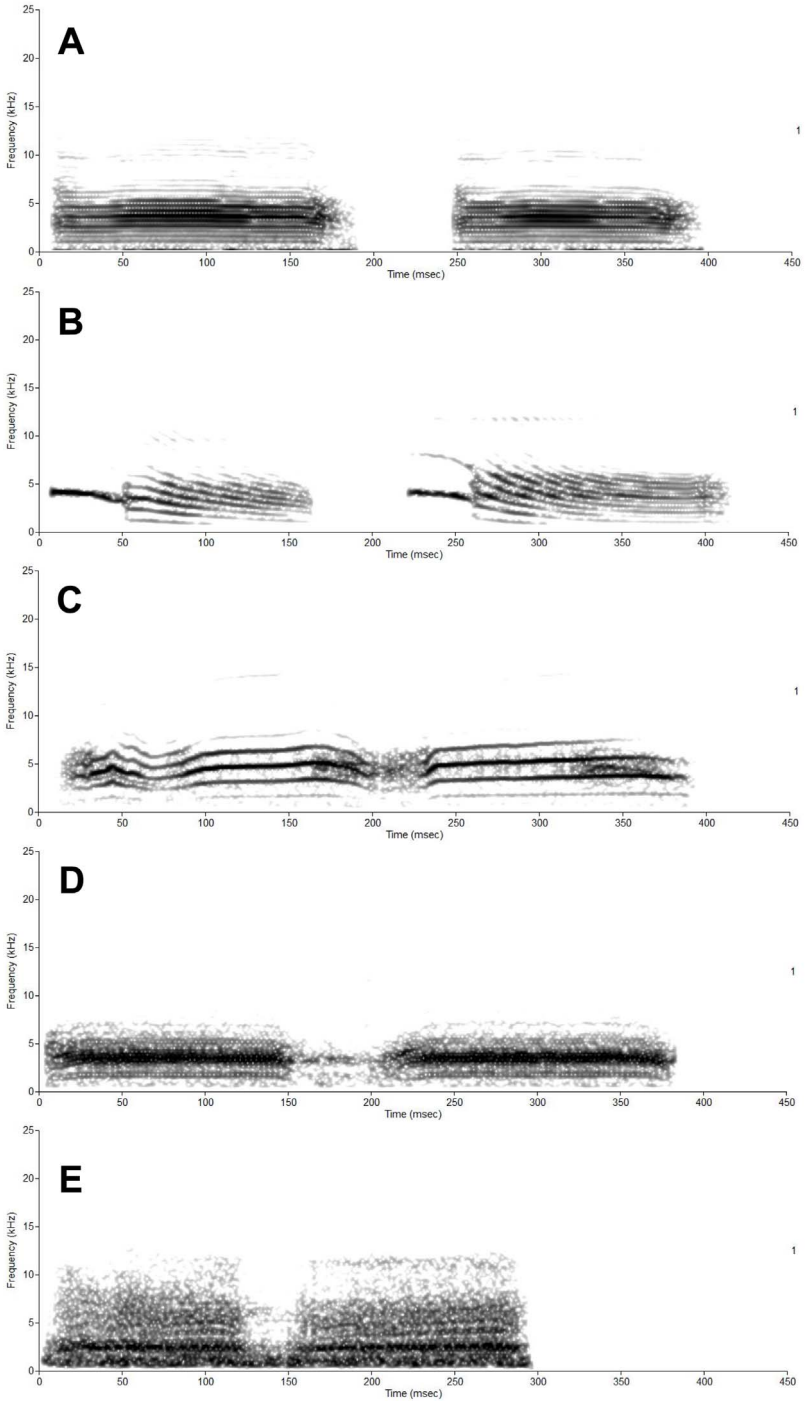

Fig. 1. Acoustic Similarity Between Stimuli. Spectrograms (transform length = 256 points; −35 to 0 dB relative to note peak amplitude) of (A) female zebra finch distance calls, (B) male zebra finch distance calls, (C) American goldfinch *tee-yee* calls, (D) black-capped chickadee *dee*-notes, and (E) tufted titmouse calls, showing acoustic similarity between the stimuli used. Each spectrogram consists of two calls or notes produced in succession by one individual.

portion of the male call, and is 2–3 times longer than an entire male call.

Unlike vocal production, the auditory pathway in zebra finches, and other songbirds, is the same in both sexes (Fig. 2). Auditory input in songbirds is initially processed by the nucleus MLd in the midbrain, which projects to the thalamus and nucleus Ov [3]. The Ov then sends auditory information to the nucleus Field L which then projects to dorsal and ventral portions of the caudomedial nidopallium (NCM) and the HVC. The NCM then sends projections to the caudomedial mesopallium (CMM) where the auditory information is processed further. After auditory input is processed in the NCM and CMM, the information is sent to vocal control nuclei HVC and robust nucleus of the arcopallium (RA) where it is further processed [4,5]. Song and call production are controlled through two interacting pathways in males that make songbirds unique compared to non-songbirds avian species.

Mello et al. [6] and Chew et al. [7] provided some evidence that the NCM is more active in response to conspecific than heterospecific vocalizations in the zebra finch. It has also been shown that female zebra finches have less neural expression in the NCM than do males following playback of both conspecific and heterospecific vocalizations [8]. In these studies [7,6,8], there was also a significant difference in neural expression in the NCM between heterospecific calls (e.g.,

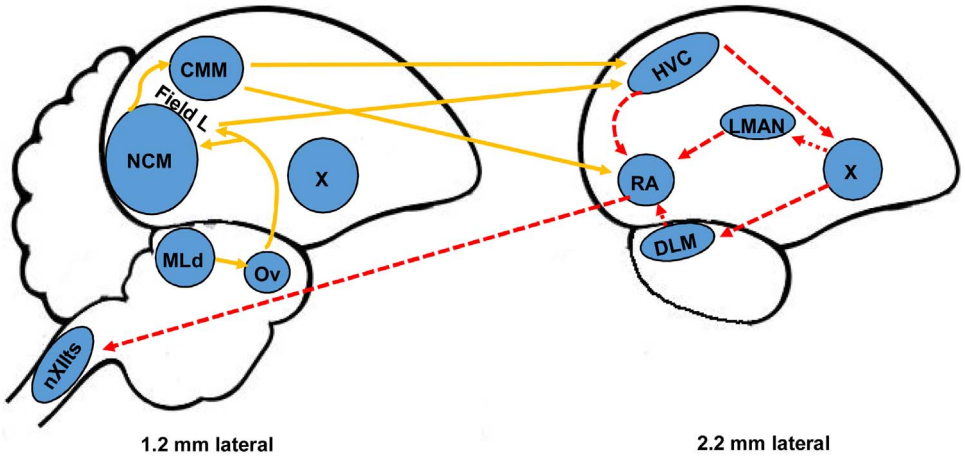

Fig. 2. Auditory and Vocalization Pathway Diagram. Schematic of neural nuclei involved in the song control pathway (dashed/red arrows) and auditory pathway (solid/orange arrows) shown on sagittal cross sections at two levels. X = Area X; CMM = caudomedial mesopallium; DLM = dorsolateral nucleus of the anterior thalamus; LMAN = lateral magnocellular nucleus of the anterior neostriatum; MLd = dorsal lateral mesencephalic nucleus; NCM = caudomedial nidopallium; nXIIts = nucleus of the twelfth cranial nerve; Ov = nucleus ovoidalis; RA = robust nucleus of the arcopallium. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

bengalese finches, *Lonchura striata*).

A common technique for characterizing neural expression in vertebrates is through visualizing the patterns and activity of immediate early genes (IEG). IEGs are genes that are rapidly transcribed after cell depolarization, with or without *de novo* protein synthesis, using the cell's preexisting transcription factors [9]. One IEG product protein, ZENK (also called Zif268, Egr-1, NGFI-A, Krox-24, TIS8), is a common tool used to visualize brain stimulation in response to various internal and external stimuli, such as in the auditory nuclei [10]. Expression of ZENK has been found to depend on multiple factors, including brain area examined, sex of the listener, and type of sound heard [11–16].

Avey et al. [1] measured ZENK expression in adult male black-capped chickadees following playback of two harmonic notes from either conspecific or heterospecific calls. Although some of the heterospecific calls (zebra finches) were produced by species more distantly related to the subjects' species compared to other heterospecific calls (chestnut-backed chickadees and tufted titmice), there was no significant difference in the amount of ZENK expression in the CMM, NCMd and NCMv following presentation of calls produced by any species (including conspecific black-capped chickadee calls). However, there was a significant difference in the average expression between auditory areas for all playback groups, with CMM having significantly more expression compared to NCMd and NCMv. These results suggest that phylogenetic distance did not influence ZENK expression in chickadees when processing hetero- and conspecific calls. This result contradicted behavioral and neurobiological results in chickadees, finches, and other songbirds illustrating the importance of phylogenetic relatedness, or how closely related species are to each other [7,8]. These neurological differences in results are most likely due to the use of acoustically similar calls by Avey et al. [1]; previous studies examining the neurological response following conspecific and heterospecific vocalizations did not use acoustically similar vocalizations e.g., [7,8].

In the current study, we used zebra finches to examine neural expression after playback of acoustically similar heterospecific and conspecific calls (Fig. 1). Assuming that the IEG response in the auditory areas observed by Avey et al. [1] was similar following playback of vocalizations produced by different species because the vocalizations were acoustically similar (rather than responding based on biological relevance), we predicted that we could replicate these previous findings using another songbird species: zebra finches. If significant differences were found between playback groups in the present study, this would suggest that, unlike black-capped chickadees, zebra finches discriminate between heterospecific and conspecific calls at the cellular level. Since zebra finches are a sexually dimorphic species, we examined ZENK expression in both males and females. We predicted that our results would reveal sex differences in expression not only due to the different composition of male and female calls, but also the different vocal production areas in the brain [17]. Exploring differences in expression between sexes would also reveal if male auditory areas are more active during a female call than a male call, a pattern observed in behavioral measurements including perch jumping and vocalizing [18].

2. Methods

2.1. Subjects and housing

This study was conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Animal Care and Use Committee for Biosciences at the University of Alberta (AUP109). Zebra finches of at least one year of age were acquired from Eastern Bird Supplies (Thetford Mines Sud, QC, Canada) and Exotic Wings & Pet Things (St. Clements, ON, Canada). Prior to use in the study, finches were housed in same-sex cages (60 cm wide × 40 cm high × 40 cm deep; Rolf C. Hagen, Inc., Montreal, QC, Canada) of up to five birds per cage, in a colony room with a 12 h light cycle, and maintained at 20 °C. All cages were in a single colony room where birds in different cages could see and hear each other, but not interact with birds in other cages. Each cage contained perches, bedding material, and opaque dividers for environmental enrichment. Food (Mazuri Small Bird Maintenance Diet; Mazuri, St. Louis, MO, USA) and water was provided *ad libitum*; twice weekly birds were provided nutritional supplementation of hard-boiled eggs with spinach or parsley.

2.2. Stimuli

We used seven types of playback stimuli. We presented male and female conspecific calls, calls produced by American goldfinch, a species that is more closely related to our study species, black-capped chickadee calls, and tufted titmice calls, which are songbirds that are more distantly related to our study species. Four acoustic properties, fundamental frequency (F_0), measured as the first visible harmonic, F_{MAX}, the maximum amplitude, and NPF, the note peak frequency, were measured according to Charrier et al. (2014) in all species calls and notes used as stimuli to examine their acoustic similarity (Table 1). As can be seen, all means (total duration, F_0, F_{MAX}, and NPF) were found to be within one standard deviation of each other. We also created reversed male and female zebra finch distance calls using SIGNAL

Table 1
Analysis of four acoustic features on notes from each species used to show acoustic similarities.

	Total Duration (ms)	F_0 (Hz)	F_{MAX} (Hz)	NPF (Hz)
Female Zebra Finch	210.2 (29.5)	436.2 (227.5)	3235.5 (437.1)	12646.2 (1307.6)
Male Zebra Finch	160.5 (46.2)	588.2 (342.2)	3367.6 (758.8)	12364.4 (1233.9)
American Goldfinch	167.4 (60.6)	562.5 (44.5)	4415.3 (1309.1)	11937.6 (2567.8)
Black-capped Chickadee	185.9 (17.7)	1624.2 (986.5)	3651.8 (245.0)	12113.0 (2745.1)
Tufted Titmouse	179.7 (31.2)	585.8 (116.4)	2683.6 (895.8)	11592.6 (1764.3)

Note: We measured all notes from each group. F_0 is the first visible harmonic, F_{MAX} is the maximum amplitude, and NPF is the note peak frequency. Averages across all measurements are shown with standard deviation in parenthesis.

software (version 5.05.02, Engineering Design, 2013; RMD and RFD, respectively). We used GoldWave (version 5.70; GoldWave, Inc., St. John's, NL, Canada) to bandpass filter all stimuli (350–1300 Hz). Each stimulus was 60 s in duration, with two separate individual calls played within the first 10 s, and approximately 5 s apart. Each stimulus was repeated on a loop for the full 30 min playback period. Five unique stimuli, containing two novel calls, were created for each playback group. Each subject was presented with a different stimulus. All stimuli were presented at approximately 75 dB as measured from the middle of the playback cage.

2.3. Playback equipment and procedure

Zebra finches were randomly assigned to one of seven groups, with three or four birds of each sex per group, for a total group size of six to seven birds per group. Playbacks were conducted in individual sound attenuating chambers (1.7m × 0.84m × 0.58m; Industrial Acoustics Corporation, Bronx, New York, USA). Birds were placed into the chambers overnight in a modified home cage (30 cm wide × 40 cm high × 40 cm deep; Rolf C. Hagen, Inc., Montreal, QC, Canada) with food and water bottles of the same color symmetrically placed on either side of the cage. The light cycle in the sound chamber was the same as in the colony room. All sessions were audio recorded using Marantz PMD670 (Marantz America, Mahway, NJ, USA) and AKG C 1000S microphones (AKG Acoustics, Vienna, Austria); we recorded 30 min of baseline during which there was no playback stimulus presented, followed by 30 min of stimulus playback. Immediately following the 30 min of stimulus playback, the chamber lights were extinguished, and a 1 h post-playback period began as peak ZENK expression is observed to occur after 60 min. After the post-playback period, each bird was anesthetized with 0.04 ml of 100 mg/ml ketamine and 20 mg/ml xylazine delivered intramuscularly (1:1). Once the bird was found to be unresponsive to a toe pinch and displaying no eye blink response, it was perfused via the left ventricle with heparinized 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA). The brain was extracted and placed in PFA for 24 h followed by a 30% sucrose PBS solution for 48 h. Brains were then fast frozen using isopentane cooled on dry ice, wrapped in foil, and stored at −80° C until sectioned.

2.4. Histology

Brains were sectioned sagittally starting at the midline into two series. The first 48 40 μm sections of each hemisphere were collected in PBS. We then processed one series of the brains (24 sections, 80 μm apart) for ZENK in batches that were randomized across the treatment groups. Sections were first washed twice in 0.1 M PBS for a minimum of 5 min each, then transferred to a 0.5% H_2O_2 solution, and incubated for 15 min, followed by three 5 min washes in 0.1 M PBS. Sections were incubated in 10% normal goat serum at room temperature for 20 h and 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 (PBS/T). Next, sections were washed 3 times (5 min each) in PBS/T before being incubated in 1:200 biotinylated goat-anti-rabbit antibody (Vector Labs, Burlington, ON, Canada) in PBS/T for 1 h. After three more washes in PBS/T, sections were incubated for 1 h in avidin-biotin horseradish peroxidase (ABC Vectastain Elite Kit; Vector Labs, Burlington, ON, Canada) and then washed three times in 0.1 M PBS. In order to visualize expression, sections were processed with 3,39-diaminobenzidine tetrachloride (Sigma FastDAB, D4418, Sigma-Aldrich, Santa Fe Springs, CA, USA) with three washes of 0.1 M PBS to remove any excess visualizing agents. Field L was used as a negative control area, as it does not express ZENK during auditory processing. In order to verify that the ZENK procedure worked correctly we included four control sections: for two sections we replaced the primary antibody (Erg-1) with PBS/T for the incubation period and for two sections we replaced the secondary antibody (biotinylated goat-anti-rabbit antibody) with PBS/T. During imaging it was noted that there was no expression in the control sections, indicating that our procedure worked.

2.5. Imaging

Eight brain sections were mounted on each slide and cover slipped. Using a Leica microscope (DM5500B; Wetzlar, Germany) with a 40 × objective, images were captured using a Retiga Exi camera (Qimaging, Surrey, BC, Canada) and Open-lab 5.1 on Macintosh OS X (Version 10.4.11). Eight images of each of the three neuroanatomical locations (CMM, NCMd, and NCMv) were collected per hemisphere, for a total of 48 images per bird. In order to ensure that there was no overlap in the images for the dorsal and ventral regions of the NCM, images of the dorsal-most and ventral-most portions of NCM were taken for all sections, as there are no distinguishing landmarks between the two areas [1]. ZENK expression was quantified by counting the number of stained cells in a representative 0.20 × 0.15 mm image using ImageJ (Fig. 3).

3. Results

We conducted a repeated measures ANOVA using SPSS (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) with brain region (CMM, NCMd, and NCMv), hemisphere (left vs. right) and section number (1–8) as within subject factors and playback condition (FDC, MDC, AGF, BCD, TTC, RMD, and RFD) and sex as between subject factors. There was a significant main effect for brain region $F(2, 104) = 40.189$, $p < 0.001$ (Fig. 4). In order to examine this significant effect, we conducted Bonferroni-corrected pairwise comparisons on brain region with an alpha level set at 0.05. This analysis found that the amount of ZENK expression in all three areas were significantly different from one another ($p < 0.01$), with CMM having the most ZENK expression ($M = 68.599 \pm 5.721$ SEM), NCMd having an intermediate amount ($M = 56.441 \pm 4.325$ SEM), and NCMv having the least ($M = 36.932 \pm 3.412$ SEM). There were no significant main effects of playback condition ($F(6, 312) = 1.853$, $p = 0.114$) or sex ($F(1, 52) = 0.706$, $p = 0.406$) or any significant interactions (see Fig. 5). While not significant, males and females had less expression in response to the black-capped chickadee *dee* notes and the tufted titmouse *dee* note calls compared to conspecific calls and the reversed calls (see

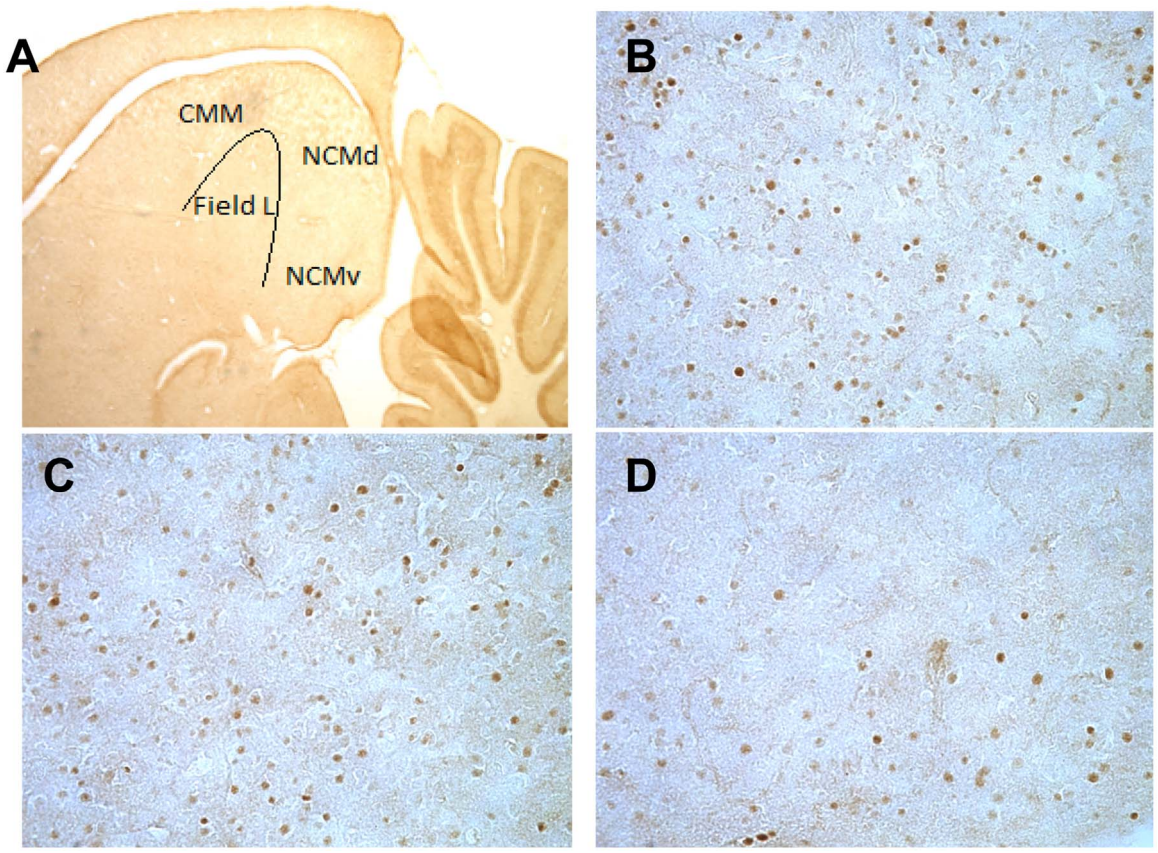

Fig. 3. Example ZENK expression in auditory areas. (A) Zebra finch telencephalon at 5× magnification. Examples of ZENK expression in the three measured areas (B) caudomedial mesopallium (C) caudomedial nidopallium, dorsal (D) caudomedial nidopallium, ventral taken at 40× magnification. All images taken from the same brain section from a male zebra finch in the male distance call playback group.

Fig. 5).

4. Discussion

Using male and female adult zebra finches, we measured ZENK expression in the CMM, NCMd, and NCMv in response to playback of conspecific and heterospecific calls produced by species of varying phylogenetic distances. We found ZENK expression in all conditions, but we found no significant differences in ZENK expression for any of the playback groups in any of the auditory nuclei measured. There was also no difference between the expression measured in males or females. However, we did find a significant difference in ZENK expression in the three auditory nuclei, with significantly more expression in CMM compared to NCMd and NCMv, and significantly more expression in NCMd compared to NCMv. This finding could indicate that, while all three nuclei are involved in auditory processing, the CMM is more active during the initial processing of auditory information. This could explain why we found more expression in the CMM.

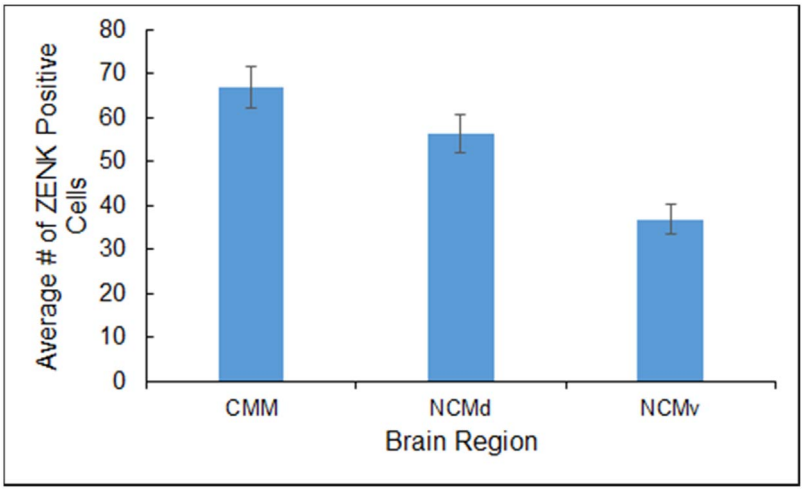

Fig. 4. Average ZENK expression by brain region. A repeated measures ANOVA showed a significant main effect of brain region across all playback groups and both sexes, $F(2, 104) = 40.189$, $p < 0.001$. A Bonferroni-corrected pairwise comparison demonstrated a significant difference between all three regions at the $p < 0.001$ level. Bars show mean ZENK expression with error bars representing the SEM; caudomedial mesopallium (CMM), caudomedial nidopallium, dorsal (NCMd), and caudomedial nidopallium, ventral (NCMv).

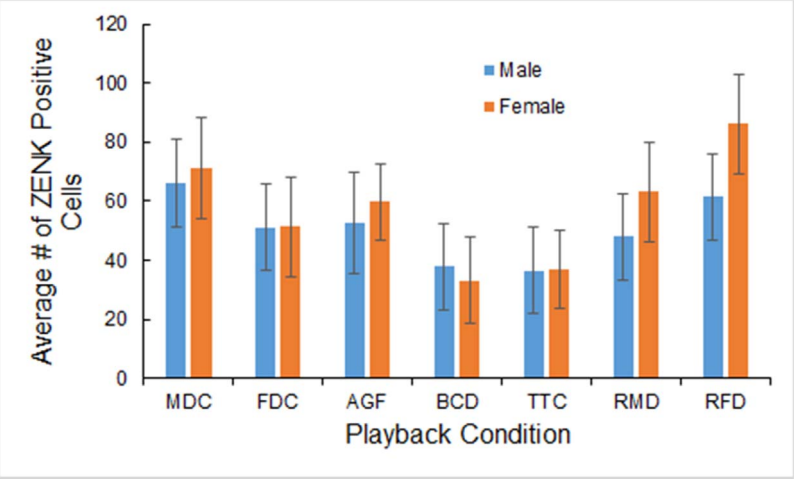

Fig. 5. Average ZENK expression by playback condition and sex for zebra finches. A repeated measure ANOVA showed that there was no significant difference in playback condition, $F(6, 312) = 0.114$, $p > 0.05$, or sex, $F(1, 52) = 0.406$, $p > 0.05$. The bar graph shows mean ZENK expression across all areas with error bars representing the SEM. Playback group names and sample sizes are; MDC: Male Distance Call (n = 4 male, n = 3 female); FDC: Female Distance Call (n = 4 male, n = 3 female); AGF: American Goldfinch Call (n = 3 male, n = 4 female); BCD: Black-capped Chickadee D-note (n = 4 male, n = 3 female); TTC: Tufted Titmouse Call (n = 3 male, n = 4 female); RMD: Reversed Male Distance Call (n = 3 male, n = 3 female); RFD: Reversed Female Distance Call (n = 4 male, n = 3 female).

Assessing later responding genes, rather than an IEG, may highlight the roles NCM plays in this auditory processing. Patterns of expression in these three areas could also be due to the type of stimuli used, as the use of song or acoustically distinct calls could produce differing levels of activation in these nuclei.

4.1. Phylogenetic relatedness

Similar to a previous study with black-capped chickadees [1], we did not find any differences in ZENK expression in response to conspecific calls and acoustically-similar heterospecific calls, regardless of differences in phylogenetic relatedness to the subject. Both the current results and those of Avey et al. [1] contradict previous studies that demonstrate that songbirds have heightened responses to conspecific vocalizations compared to heterospecific vocalizations at the neurological level. Previous behavioral data have shown that both black-capped chickadees [19] and zebra finches [20] will respond more to vocalizations of their own species than to those of other species. This could suggest that while these birds are able to differentiate between heterospecific calls and conspecific calls [21], they are simply doing so further along the auditory pathway than in auditory areas CMM, NCMd, or NCMv, or at subsequent stages of processing not assessed here or elsewhere.

The lack of significant differences observed between playback groups is also in contrast to previous electrophysiological evidence. Chew et al. [7] found significant differences in firing rates recorded in the NCM between the heterospecific playbacks of biologically relevant calls (canaries, *Serinus canaria*; bengalese finches, *Lonchura striata*; and silverbills, *Lonchura malabarica*), and between all heterospecific and the conspecific playback of zebra finches calls when measuring extracellular activity using *in vivo* recordings. [22] also found significant activity in male zebra finch NCM and Field L in response to conspecific vocalizations using fMRI. While this previous study analyzed NCM overall, in the current study we examined the dorsal and ventral sections of the NCM separately. By analyzing the dorsal and ventral sections separately, we were able to accurately identify which regions in the NCM were active during playback to determine if there was a certain portion of NCM that specialized in species discrimination. Chew et al. [7] used calls produced by species that were acoustically dissimilar to the species under study (zebra finches) and produced by species that zebra finches would likely encounter in the wild; however, in the current study, we used calls produced by heterospecific species that are acoustically similar to the zebra finch distance call. Another key difference is that ZENK expression was analyzed 1 h after playback while Chew et al. recorded extracellularly in awake finches and assessed responsiveness online. Previous studies have shown that there are differences between cellular activity and ZENK gene expression [23]. It is thought that, while many areas are active during auditory processing, only areas involved in modulating long-lasting cellular changes, such as long-term memory, will express the ZENK gene [6,5]. Future research should analyze differences in extracellular activity to phylogenetically close or distantly related heterospecific calls, as ZENK cannot measure activity patterns.

Male zebra finches have been described as using a multidimensional, or step-by-step, approach to discriminate stimuli [18]. By being able to compare novel calls heard to learned calls produced from their tutors when young, male zebra finches can categorize call-based dimensions such as call duration and fundamental frequency [18]. In the current study, we presented zebra finches with acoustically similar calls produced by heterospecific individuals, so birds may have experienced greater difficulty in discriminating among call stimuli. In contrast, previous studies that have found differences in neural activity to conspecific and heterospecific vocalizations have only considered the phylogenetic relatedness of the vocal producer to the subject species, but did not consider the influence of the acoustic structure of the vocalizations (e.g., [7,24]; but see [1].

Overall, our results examining the response to acoustically similar heterospecific and conspecific calls in zebra finches are consistent with the previous findings in black-capped chickadees [1]. However, one notable exception is the neural response to the control conditions (i.e., the reversed vocalizations). In the current study, we found no significant difference in the amount of expression following playback of the reversed vocalizations compared to the forward vocalizations; however, Avey et al. [1] reported significantly less expression following reversed notes compared to the other playback conditions. There are two differences between this study and Avey et al. [1] that may have caused this disparity: the number of controls and species used. Using two controls (a reversed male distance call and a female distance call) could have had different effects on either male or female birds. However, when we examined the sexes separately, there was still no difference between the experimental and control groups. With regard to study species, there are many behavioral and ecological differences between black-capped chickadees and zebra finches. However, our results suggest a difference that has not been examined previously.

What made the controls effective in the study by Avey et al. [1] was the importance of syntax, or note order, to chickadees [25]. It is possible that zebra finches do not pay as much attention to syntax as chickadees, making the controls less effective. The use of syntax by songbirds has also been shown for other species, such as the Japanese great tit (*Parus minor*), which is more closely related to the black-capped chickadee than to the zebra finch [26]. This could then suggest that the ability to use syntax, a behavior previously thought to occur only in humans, could be phylogenetically recent, or a trait newly evolved, in some species of songbirds. It is important to note that while there was a significant decrease in the expression to the reversed D notes in Avey et al. [1], there was still a high amount of expression to this stimuli. Future studies could use silence or white noise as a control to show that the birds are responding differently when presented any stimuli than when at rest.

4.2. Sex differences

Contrary to our hypothesis that there would be a difference in neural expression between sexes, male and female zebra finches did not differ significantly in patterns or amount of ZENK expression in response to heterospecific calls or conspecific calls, suggesting the auditory system of males and females respond in a similar fashion when exposed to these calls. Since we found no difference among playback groups, male and female zebra finches may assess the species of an individual producing a call in the same brain areas. Even though it is technically part of the song system, RA has been shown to play an important role in the ability of zebra finches to identify the sex of a conspecific vocalizer [27]. Future studies should examine RA to determine if there is a difference in response to male and female conspecific calls, or a difference in response to calls produced by heterospecifics.

Using another zebra finch vocalization, the long call, Vicario et al. [18] found that male zebra finches vocally respond more to female long calls than to male long calls providing evidence that zebra finches can discriminate between male and female calls. Vicario et al. [18] also found that female zebra finches responded more to female than to male calls, though it is thought that both sexes responded to acoustic structure (in this case, length of the call) rather than the sex of the vocalizer. However, our results are similar to previous studies looking at ZENK expression between sexes. Both the zebra finch song and long call have been shown not to produce differing levels of ZENK expression between sexes [28–30]. In black-capped chickadees, it has been suggested that a given brain area, such as the NCM, will express different amounts of ZENK expression in response to different vocalization types (e.g., a gargle or a *chick-a-dee* call), which could also explain differences that are seen in the zebra finches [11,15]. Acoustic structure may have also played a role in the present study, as neither males nor females responded differentially to heterospecific calls with acoustic similarity to conspecific distance calls.

4.3. Conclusion

As predicted, we found no difference in neuronal expression of ZENK in the auditory pathways of zebra finches in response to acoustically similar calls produced by both conspecific and heterospecific songbirds of differing degrees of phylogenetic relatedness. Our findings nearly paralleled those of Avey et al. [1]; taken together, the results of both studies suggest that identification of the species of vocal callers may not be processed in the auditory pathway in songbirds. The lack of significant differences between experimental and control groups in the current study may suggest that zebra finches do not attend to syntax similar to other species of songbirds.

Contrary to our prediction, there was no difference between sexes in ZENK expression regardless of the identity of the call's producer. Similarity in the duration and acoustic features of all calls used in this experiment may have played a role in the inability of both male and female zebra finches to distinguish heterospecific calls from conspecific calls. Further research examining responses in the song system nuclei, which receive feedback from the auditory nuclei, may be the key to identifying where zebra finches process information about the identity of vocalizers.

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