A re-examination of ZENK expression following hetero- and conspecific playback in the zebra finch auditory forebrain

by

Erin N Scully

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Department of Psychology
University of Alberta

Abstract

Zebra finches (*Taeniopygia guttata*) are one of the most sexually dimorphic songbirds used as model species, not only in appearance but also in vocal production; while males produce both calls and songs, the females only produce calls. This dimorphism in the zebra finch 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, i.e., the black-capped chickadee (*Poecile atricapillus*). In this study I looked at neuronal expression after playback of acoustically similar hetero- and conspecific calls in male and female zebra finches, as a follow-up study to previous work conducted by Avey and colleagues (2014) on black-capped chickadees. An immediate early gene (IEG), ZENK, was measured in two auditory areas of the forebrain (caudomedial mesopallium, CMM, and caudomedial nidopallium, NCM). In black-capped chickadees, there was no significant difference in expression for calls produced by other species that were phylogenetically distant. In the current study, I found no difference in ZENK expression in either male or female zebra finches regardless of playback conditions. My results suggest that, similar to black-capped chickadees, zebra finch IEG expression in the CMM and NCM is related to the acoustic similarity of vocalizations and not the phylogenetic relatedness of the species producing the vocalizations.

Preface

This thesis is an original work by Erin Nicole Scully. No part of this thesis has been previously published. All procedures followed the Canadian Council on Animal Care (CCAC) Guidelines and Policies and were approved by the Animal Care and Use Committee for Biosciences at the University of Alberta (AUP 108). I was responsible for designing the concept, collection of data, data analysis, and manuscript composition. N. McMillan provided manuscript edits. C. B. Sturdy was the supervisory author and was involved with the formation of concepts and revision of the manuscript.

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List of Abbreviations

AGF- American goldfinch call

BCD- Black-capped chickadee *dee*-note

CMM- Caudomedial mesopallium

DAB- 3,3′-Diaminobenzidine

DLM- Dorsolateral nucleus of the anterior thalamus

FDC- Female zebra finch distance call

ICo- Nucleus intercollicularis

IEG- Immediate early gene

IMAN- Nucleus magnocellularis of the anterior neostriatum

MDC- Male zebra finch distance call

NCM- Caudomedial nidopallium

NCMd- Caudomedial nidopallium, dorsal

NCMv- Caudomedial nidopallium, ventral

PBS- Phosphate buffered saline

PBS/T- Phosphate buffered saline with Triton X-100

PFA- Paraformaldehyde

RA- Robust nucleus of the arcopallium

RFC- Reverse female distance call

RMC- Reverse male distance call

SEM- Standard error of the mean

TTC- Tufted titmouse call

Introduction

Songbirds, birds classified as Oscine Passeriformes, are some of the most common birds on Earth (Mayr, 1946). These birds can be found across the globe, in multiple climates, and in many different colors and sizes ranging from large ravens and crows to small finches and chickadees. Songbirds are best known for their complex vocalizations owing to their complex vocal control organ, the syrinx (Catchpole & Slater, 2008). In many species of songbirds, young learn their vocalizations through the use of a tutor, similar to how human infants mimic adults to learn speech. This is different from the majority of animals that do not learn their vocalizations.

Songbirds produce two categories of vocalizations: calls and songs. Songs, produced mainly by males in temperate songbirds, are used as a way to attract mates, as well as a way to repel competing birds from claimed territories. Calls are produced by both sexes, and have multiple functions including alerting other birds to food, predators, and conspecific locations. While these two types of vocalizations have distinctly different purposes, the same neural areas are used in their production.

The songbird song-system, an interconnected set of brain areas possessed by songbirds, is necessary in producing not only songs, but calls as well (see Figure 1; also see Ball & MacDougall-Shackleton, 2001). There are two pathways controlling vocal production. The main pathway starts in the telencephalon, with the HVC (proper name) which projects to the robust nucleus of the arcopallium (RA) that projects to the nucleus intercollicularis (ICo) and the twelfth cranial nerve (XIIts). Projections are then sent from XIIts to the syrinx where they synapse with muscle fibers (Nottebohm et al., 1976). There is a second pathway that is anterior to the main pathway that also starts at HVC, which is thought to be more involved in song learning, maintenance, and behavior modification (Brenowitz, 1991). Here, the projections travel

from HVC to Area X, which then projects to the dorsolateral nucleus of the anterior thalamus (DLM), that connects to the nucleus magnocellularis of the anterior neostriatum (IMAN), which projects to RA, projections then continue in the main production pathway (Ball & MacDougall-Shackleton, 2001).

In turn, auditory input is first processed by the nucleus MLd in the midbrain, which projects to the thalamus and nucleus Ov (Mello et al., 2004). 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 in processed further. After auditory input is processed, the information is then sent to vocal control nuclei HVC and RA where it is further processed (Matragrano et al., 2012; Mello & Clayton, 1994). While other birds also have some of these vocal and auditory areas, the two vocal production pathways and the interaction with the auditory pathway in songbirds contributes to their unique vocal abilities.

Many songbird species display sexual dimorphism in song production as well as song nucleus size. In the majority of these species exhibiting dimorphism, males produce more complex songs than females, as well as more song types, and are found to have larger song nuclei, up to five times larger in species such as the zebra finch (*Taeniopygia guttata*; Ball et al., 1995; Ball et al., 1994; Gahr et al., 1998; Kirn et al., 1989). Voigt and Gahr (2011) found that the HVC of subordinate males and females in the white-browed sparrow weaver (*Plocepasser mahali*) are similar in phenotype, though females have fewer cells in the HVC. In species such as the streak-backed oriole (*Icterus pustulatus*), where females produce just as complex, or even more complex, songs than, females still have smaller HVC and area X, but not RA (Hall et al., 2010). Rufous-and-white wrens (*Thryothorus rufalbus*) have larger song nuclei than close

relatives, the bay wren (*Thryothorus atricapillus*), even though females of both species produce similar songs (Brenowitz & Arnold. 1986). In black-capped chickadees (*Poecile atricapillus*), females have been found to produce songs with a similar acoustic structure as males, although females produce songs with a larger frequency decrease in the first note (Hahn et al., 2013). While area X is smaller in female black-capped chickadees relative to males, there is no sex difference observed in HVC or RA, in birds in breeding or nonbreeding conditions (Phillmore et al., 2014).

Zebra finches are sexually dimorphic, with only the males producing a song. During development, males use tutors to learn quality songs, while females use tutors to learn song preferences (Zann, 1996). Researchers have found that while the male distance call, used for identity, alarm, and localization, is controlled by the same neural pathway as song production, the female call is controlled by a simpler pathway in the brain stem that also controls respiratory patterning (Simpson & Vicaro, 1990; Vicario et al., 2001a; Zann, 1996). It has also been shown that during development, males initially use a simpler pathway than females use, and will revert back to it if the song system pathway is severed (Vates et al., 1996).

The main call of the zebra finch is the distance call, which is made up of two components: a tonal portion which has complex harmonics and ends with a higher frequency, and a noise (broadband) portion that is characterized by a downward sweep of the harmonics that gives a more grating sound (Zann, 1996). Each call may contain differing numbers of tone and noise notes in any order, although one tone note followed by one noise note is most common. While both male and female zebra finch produce this call, as shown in Figure 2, they differ in call composition and duration, with the female call typically being longer and less complex than the male call. In the male call, the two components can easily be seen, starting with a higher

frequency portion that then swoops down to a longer, more harmonic portion of slightly lower frequency (Figure 2). The female call, on the other hand, only has one distinct frequency, which is most similar to the second portion of the male call, and can last 2-3 times longer than the male call.

Many animal vocalizations are used to find a potential mate, and produce offspring to continue the genetic lineage. It has been shown often that songbirds are able to distinguish among individuals within their own species, but it is also important for them to recognize species identity as well. Many animals have been shown to have the ability to differentiate between conspecific and heterospecific songs and calls, from songbirds to frogs to monkeys (Dooling et al., 1992; Ryan & Rand, 1995; Brown et al., 1994). While it may be easy for songbirds to distinguish between a songbird and a frog, it is more difficult for them to distinguish between two phylogenetically close songbird species (Ryan & Rand, 1995). The black-capped chickadee has recently been shown to not only respond to conspecifics, but also to closely related heterospecifics, the chestnut-backed chickadee (*Poecile rufescens*) and the tufted titmouse (*Baeolophus bicolor*; Hahn et al., 2016).

While many previous studies have looked at the behavioral response to heterospecific calls, there has been little research on the neural response. Chew et al. (1996) 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 produce slightly less expression than males in the NCM to both conspecifics and heterospecifics (Yoder et al., 2014). In both of these studies, there was also a significant difference in neural expression in the NCM between heterospecific calls of biologically relevant species.

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 activation, with or without *de novo* protein synthesis, using the cell's preexisting transcription factors (Watson & Clements, 1980). These IEGs can code for multiple things, including more transcription factors, functional and structural proteins, and signaling molecules (Okuno, 2010). While IEGs were originally found in the central nervous system, they have recently also been found in multiple types of cells throughout the body (Okuno, 2010; Watson & Clements, 1980). Visualization of these IEGs is often done using immunohistochemistry, by attaching a series of antibodies and visualizing agents (fluorescent markers, 3,3′-Diaminobenzidine, etc.) to the gene product.

Zif268/Egr-1/NGFI-A/Krox-24/TIS8/ZENK, all different names for the same IEG product protein, is a common tool used to visualize brain stimulation in response to various external stimuli in the auditory nuclei (Knapska & Kaczmarek, 2004). This IEG is a transcription factor protein that can be visualized by specific expression patterns unique to this gene (Cao et al., 1990; Milbrandt, 1987; Waters et al., 1990). While its specific function is unknown, it has been suggested that it is involved in long-term memory formation, synthesis of proteins used in song production, and synthesis of proteins involved in motor memory of song learning (Jarvis & Nottebohm, 1997; Mello et al., 1992). ZENK, the avian form of this gene, is expressed with peaks 90 minutes after exposure to stimuli, allowing time for specimens to be collected/preserved for future visualization.

Avey and colleagues (2014) measured ZENK expression in adult male black-capped chickadees (a sexually monomorphic species) after playback of calls, from species both phylogenetically close and distant, composed of two harmonic notes. They used five groups: one

conspecific call, three heterospecific calls, and one reversed conspecific call to act as a control as syntax is known to be important to chickadees. They found that there was no significant difference in the amount of ZENK expression in the caudomedial mesopallium (CMM) and caudomedial nidopallium (NCM; dorsal and ventral) for any group, except for the control, the reversed conspecific calls. However they did find significant difference in average expression between auditory areas for all auditory groups. This therefore suggested that phylogenetic distance did not influence ZENK expression in chickadees when processing con- and heterospecific calls. This was important, as it contradicted what was previously shown behaviorally in multiple species, as well as neurologically in other songbirds (Chew et al., 1996; Yoder et al., 2014). These differences are most likely due to the use of acoustically similar calls by the Avey group (2014), which was not the case in the other studies.

I predicted that I could replicate, in zebra finches, the findings of Avey and colleagues (2014) for black-capped chickadees, specifically that there would be no difference in response between playback groups but a difference between auditory areas responding to acoustically similar calls rather than biologically relevant calls as previous studies have examined. I chose to use zebra finches because their behavior is very generalizable to other songbird species due to their call structures and auditory structure. They are also, along with canaries (*Serinus canaria*), one of the first songbirds in which hetero- verses conspecific call discrimination was studied (Mello et al., 1992). Zebra finch calls have harmonic structures, they were one of the first species shown to discriminate between heterospecific and conspecific calls, and their auditory system has sensitivity relatively similar to other songbirds (Dooling et al., 1992; Zann, 1996). If I found a significant difference between playback groups, this would suggest that unlike the black-capped chickadee, zebra finches discriminate between conspecific and heterospecific calls at the

cellular level. Since zebra finches are a sexually dimorphic species, I looked at potential differences between sexes as well as between the responses to different playbacks. I used both positive and negative controls in my study. For positive controls I used reversed calls, as these calls are expected to produce some ZENK expression in the three areas, but less expression than normal, forward calls. My negative controls were brain sections not treated with the full ZENK procedure, to show that each step of my procedure worked correctly. I also used Field L as a type of negative control area, as it is known not to express ZENK during auditory processing, even when the procedure is done correctly. I predicted that my results would reveal sex differences in expression due to not only different composition of calls, but also different vocal producing areas in the brain (Vates et al., 1996). Exploring differences in expression between sexes would also help reveal if male auditory areas are more active during a female call than a male call, a pattern seen in behavioral data, such as perch jumping and vocalizing (Vicario et al., 2001b).

Methods

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 for the University of Alberta (AUP109). Zebra finches were acquired from Eastern Bird Supplies (Thetford Mines Sud, QC, Canada) and Exotic Wings & Pet Things (St. Clements, ON, Canada) were at least 1 year of age. Prior to use in the study, finches were housed in same-sex cages (60 cm wide x 40 cm high x 40 cm deep; Rolf C. Hagen, Inc., Montreal, QC, Canada) of up to five birds, in a colony room with a 12 hour light cycle, and maintained at 20 degrees Celsius. All cages were in the same colony room were birds in different cages could see and hear each other, but not interact. All cages contained perches, bedding material, and covers to hide behind for

environmental enrichment. Food (Mazuri Small Bird maintenance Diet; Mazuri, St. Louis, MO, USA) and water was provided at all times, as well as supplementation of hard-boiled eggs with spinach or parsley twice a week.

Stimuli

Stimuli were in accordance with Avey et al. (2014). I used the same stimuli from the previous study for four of the playback groups: female zebra finch distance call (FDC), male zebra finch distance call (MDC), black-capped chickadee *dee*-notes (BCD), and tufted titmouse call (TTC). All new stimuli were created using the same programs and procedures as used to create the original stimuli by Avey et al. (2014). I used SIGNAL software to create the reversed male and female zebra finch distance calls (RMD and RFD, respectively), and GoldWave version 5.70 (GoldWave, Inc., St. John's, NL, Canada) to bandpass filter the calls (350-1,300 Hz). The remaining group of stimuli created for the American goldfinch (*Spinus tristis*) call (AGF), were created using GoldWave and SIGNAL to bandpass filter and standardize the amplitude of the calls and to add 5 ms at the beginning and end of each vocalization tapered. Each stimulus was 1 min in length, with two separate individual calls played within the first 10 sec, and approximately 5 sec apart. Each stimulus was repeated on a loop for the full 30 min playback period. I presented all stimuli at approximately 75 dB.

Playback Equipment and Procedure

Zebra finches were randomly assigned to one of seven groups, with 3-4 birds of each sex per group, for a total group size of 6-7 birds per group. Playbacks were conducted in individual sound attenuating chambers (1.7m x 0.84m x 0.58m; Industrial Acoustics Corporation, Bronx, New York, USA). Birds were placed in the chambers overnight in a modified home cage (30 cm wide x 40 cm high x 40 cm deep; Rolf C. Hagen, Inc., Montreal, QC, Canada) with food and

water bottles of the same color placed at the same location on either side of the cage. The light cycle in the sound chamber was the same as in the colony room. A pre-playback baseline of 30 min of silence was followed by a 30 min playback session. Audio and video recordings of these sessions were obtained using Marantz OMD670 cameras (Marantz America, Mahway, NJ, USA) and AKG C 1000S microphones (AKG Acoustics, Vienna, Austria). Following the playback session, the chamber lights were turned off, and a 1 hour post-playback period began. After the 1 hour post-playback period, each bird was overdosed with (0.04 ml) of 100 mg/ml ketamine and 20 mg/ml xylazine intramuscularly (1:1). Once the bird was found unresponsive to both a toe pinch and 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 then removed and placed in PFA for 24 hours before being placed in a 30% sucrose PBS solution for 48 hours. Brains were then fast frozen using isopentane in dry ice, wrapped in foil, and stored at -80° C until sectioned.

Histology

Brains were sectioned sagittally starting at the midline into two series. The first 48 40µm sections of each hemisphere were collected in PBS. I then processed the brains 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 minutes each. Sections were then transferred to a 0.5% H_2O_2 solution, and incubated for 15 minutes, followed by three 5 min washes in 0.1 M PBS. Brains were then incubated in 10% normal goat serum at room temperature for 20 hours. They were then transferred into the primary antibody (erg-1, catalogue # sc-189, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 24 hours at a concentration of 1:5,000 in 0.1 M PBS with Triton X-100 (PSB/T). Three washes followed in PBS/T before being incubated in 1:200 biotinylated

goat-anti-rabbit antibody (Vector Labs, Burlington, ON, Canada) in PBS/T for 1 hour. After three more washes in PBS/T, sections were incubated for 1 hour in avidin-biotin horseradish peroxidase (ABC Vectastain Elite Kit; Vector Labs, Burlington, ON, Canada). Sections were then washed three times in 0.1M PBS. In order to visualize expression, sections were then processed with 3,39-diaminobenzidine tetrachloride (Sigma FastDAB,D4418, Sigma-Aldrich, Santa Fe Springs, CA, USA) with three washes of 0.1M PBS to remove any excess visualizing agents. In order to verify that the ZENK procedure work correctly, I ran two sections through the normal procedure, however instead of using the primary antibody, Erg-1, the sections was placed in PBS/T for the incubation period. Another two sections were also used as controls, this time replacing the secondary antibody, biotinylated goat-anti-rabbit antibody, with PBS/T.

Imaging

Eight cross-sections were mounted on each slide with cover slips. Using a Leica microscope (DM5500B; Wetzlar, Germany), I captured images of the cross-sections to quantify the amount of ZENK expressed in each region of the brain. Each of the images were captured under 40x objective and a Retiga Exi camera (Qimaging, Surrey, BC, Canada) using 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. There was no overlap in the images for the dorsal and ventral regions of the NCM.

Statistical Analysis

ZENK expression was measured by counting the number of stained cells in a representative 0.20x0.15mm image (Figure 5). Expression was measured using ImageJ for the first eight sections of both hemispheres for each of the three Brain regions of interest. I then used SPSS (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) to perform a

repeated measures ANOVA for each Playback Condition by Sex for the between subject factor, and a repeated measures ANOVA for each Brain region (CMM, NCMd, and NCMv) by hemisphere (left vs right) and section number (1-8) for the within subject factor. I also ran a Bonferroni-corrected pairwise comparison on Brain region. All tests of significance used an alpha level of 0.05.

Results

Similar to Avey et al. (2014), ZENK expression was present in all three auditory areas, CMM, NCMd, and NCMv, for each of the seven Playback Conditions (FDC, MDC, BCD, TTC, RMD, and RFD; Figure 6). I found no interaction effects between any of my variables (Figure 4). I found a main effect for Brain Region (F (2, 104) = 40.189, $p < 0.001$; Figure 3). The pairwise comparisons for Brain Region 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 (Mean 68.599 ± 5.721 standard error of the mean (SEM)), NCMd having an intermediate amount (Mean 56.441 ± 4.325 SEM), and NCMv having the least (Mean 36.932 ± 3.412 SEM). Unlike the previous study, I did not find any main effects of Playback Condition (F (6, 312) = 0.114, $p > 0.05$; Figure 5). The repeated measures ANOVA also did not find any significant effect of Sex (F (1, 52) = 0.406, $p > 0.05$; Figure 5) or any significant interactions. While not significant, both males and females did appear to have less expression in response to the black-capped chickadee D notes and the tufted titmouse call compared to conspecific calls and the reversed calls.

Discussion

Using both male and female adult zebra finches, I measured immediate early gene, ZENK, expression in the CMM, NCM dorsal, and NCM ventral nuclei in response to playback of conspecific and heterospecific calls of varying phylogenetic distances. In all conditions, we found ZENK expression. I found no significant differences between 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, regardless of playback condition. I did find that there was a significant difference in ZENK expression between the three auditory nuclei measured, with CMM having the most, followed by NCMd, and NCMv having the least.

Phylogenetic Relatedness

Similar to research utilizing black-capped chickadees (Avey et al., 2014), I did not find any differences in ZENK expression in response to acoustically similar, heterospecific calls, regardless of phylogenetic relatedness, nor to conspecific calls. I also found that the three auditory areas that I measured had significantly different expression levels from one another, again similar to Avey et al. (2014). This could indicate that while all three nuclei are involved in audition, the CMM is more active during the initial processing of information, with more detailed processing that requires longer taking place in the NCM afterwards. This could explain why I found more activity in the CMM, and perhaps if I waited longer before perfusion, there would be more activity in the NCM instead. This could also be due to the type of stimuli I used, and use of a song or acoustically distinct calls could produce differing levels of activation in these nuclei.

Both my results and those of Avey et al. (2014) contradict evidence of species differentiation in the auditory system of songbirds. Previous behavioral data have shown that both black-capped chickadees and zebra finches will respond more to vocalizations of their own

species than to those of heterospecific individuals (Charrier & Sturdy, 2005). This could suggest that while these birds are able to differentiate between heterospecific calls and conspecific calls, they are simply not doing so in the auditory areas CMM, NCMd, or NCMv, but perhaps further down the auditory pathway.

The lack of differences between playback groups also contradicts previous electrophysiological evidence. Chew et al. (1996) found significant differences in the NCM between their heterospecific groups of biologically relevant calls and the conspecific group of zebra finches calls when measuring extracellular activity in live birds. However, I split the NCM into the dorsal and ventral sections, whereas Chew and colleagues analyzed it as a whole. By analyzing the dorsal and ventral section separately, I was able to accurately identify which regions in the NCM were active during my playback calls, and if there was a certain area that specialized in species discrimination, which I did not find. A key difference that separates my study from Chew et al. (1996) is that I was using the calls of heterospecifics that are acoustically similar to the zebra finch distance call, while the previous paper was using calls of birds that zebra finches would be likely to encounter in the wild. Another key difference is that I analyzed ZENK expressed 1 hour after playback while Chew and colleagues (1996) recorded extracellularly in awake finches. It is important to note that previous studies have shown that there is a difference in cellular activity and ZENK gene expression. 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 (Mello et al., 1992; Mello & Clayton, 1994). My results could mean that while the NCM is active and responding differently to conspecific and heterospecific calls, ZENK expression does not respond differentially.

Male zebra finches have also been described as using a multidimensional approach to discriminate stimuli (Vicario et al., 2001b). By being able to compare calls heard to those learned from their tutors when young, male zebra finches can categorize call-based dimensions such as call duration and fundamental frequency. Because my study presented birds with acoustically similar calls produced by heterospecific individuals, zebra finches may have experienced greater difficulty in discriminating among call stimuli compared to other studies that only considered phylogenetic relatedness, and did not incorporate call structure (Chew et al., 1996; Vicario et al., 2001a; Vicario et al., 2001b). More complex processing of auditory information may occur further downstream from the CMM and NCM, which allows zebra finches, and possibly chickadees, to distinguish between acoustically similar stimuli.

My results largely mirrored those of Avey et al. (2014), except for results for controls. Avey et al. (2014) and I examined ZENK expression in the CMM and NCM (dorsal and ventral) in response to acoustically similar heterospecific and conspecific calls. Both studies used reversed conspecific calls as controls, but only Avey et al. (2014) reported significant differences among experimental groups. There are two differences between the studies that may have caused this disparity: number of controls and the species used. Using two controls, one a reversed male distance call and one a female distance call, could have had different effects on either male or female birds. However, when I examined the sexes separately, there was still no difference between the control groups and the experimental groups. With regard to study species, there are many differences between black-capped chickadees and zebra finches, behaviorally and ecologically. However, my results suggests a difference that has not been examined previously. What made the controls effective in the study by Avey et al. (2014) was the importance of syntax to chickadees (Lucas & Freeberg, 2007). It is possible that zebra finches do not pay as much

attention to syntax as chickadees do, making my positive 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 the zebra finch (Suzuki et al., 2016). This could then suggest that the ability to use syntax, a behavior previously thought to occur only in humans, could be phylogenetically recent in some species of songbirds.

Sex Differences

Contrary to my hypothesis that there would be a difference in neural expression between sexes, male and female zebra finches did not differ significantly in patterns of ZENK expression for heterospecific calls or conspecific calls. The auditory system of males and females may have responded in a similar fashion when exposed to these calls. As I found no difference among playback groups, both 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, region RA has been shown to play an important role in the ability of zebra finches to identify the sex of a conspecific vocalizer (Vicario et al., 2001a). Future studies should examine RA to see if perhaps there is a difference in response to sex of the conspecific calls, as well as a clue to where discrimination of heterospecific calls may take place.

Another type of zebra finch call, the long call, has also been used to compare how males and females respond to conspecific calls. Vicario et al. (2001b) found that male zebra finches will respond more to female long calls than male calls. While this study only collected behavioral data, it suggests that male finches in my study should have exhibited more neural expression to the female distance call than the male call; however, I did not find this. Acknowledging that my experiment used a different call type, it suggests that nuclei outside of the auditory pathway should be examined, such as the RA. Vicario et al. (2001b) also found that

female zebra finches responded more to female calls, though it is thought that they responded to the length of the call rather than the sex of the vocalizer. Call structure may have played a role in the present study as females did not respond differentially to heterospecific calls which were similar in length to their own distance calls.

Limitations & Future Directions

A possible shortcoming of my study was, in order to obtain a similar acoustic structure, I did not use the full calls of some heterospecific birds, specifically the black-capped chickadee call. Perhaps if I had used the full call, there would have been a significant difference in ZENK expression in the auditory nuclei compared to the conspecific call. Based on my findings, further research is needed to understand fully where and how zebra finches discriminate between the acoustically similar vocalizations produced by different species. Since I did not find any differences in the CMM or NCM, looking further down the auditory pathway, at RA and HVC, may show ZENK expression differences in response to heterospecific and conspecific calls. A similar methodology could be used, measuring ZENK expression in the RA and HVC in response to heterospecific and conspecific calls that share acoustic features.

The lack of difference between my control and experimental groups suggest that the zebra finch does not use syntax as a few other songbirds have been shown to do. Another future direction would be to look more closely at the organization of zebra finch vocalizations. It would be interesting to look beyond the distance call, to other calls and the song of the zebra finch, to see if birds are able to discriminate between natural and synthetic vocalizations. Synthetic vocalizations could be full vocalizations played in reverse, as in my study, or subsets of notes or components of notes. Future studies could use silence or white noise as a control, to ensure that the birds are responding differently when presented stimuli than when at rest; this was not

possible with in the current study design. Use of silence or white noise as the control condition could be done using an operant conditioning task, in conjunction with measurement of neural expression in response to auditory playback.

Conclusion

As predicted, I found no difference in neuronal expression of ZENK in the auditory pathway of the zebra finch in response to acoustically similar calls produced by both conspecific and heterospecific songbirds of differing degree of phylogenetic relatedness. My findings nearly parallel those of Avey et al. (2014), which suggested 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 the zebra finch does not attend to syntax as do some other species of songbirds.

Contrary to my prediction, there was no difference between sexes in ZENK expression regardless of the identity of the call's producer. Similarity in the lengths and acoustic features of all calls used in my experiments 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 key to finding where zebra finch process information concerning the identity of vocalizers.

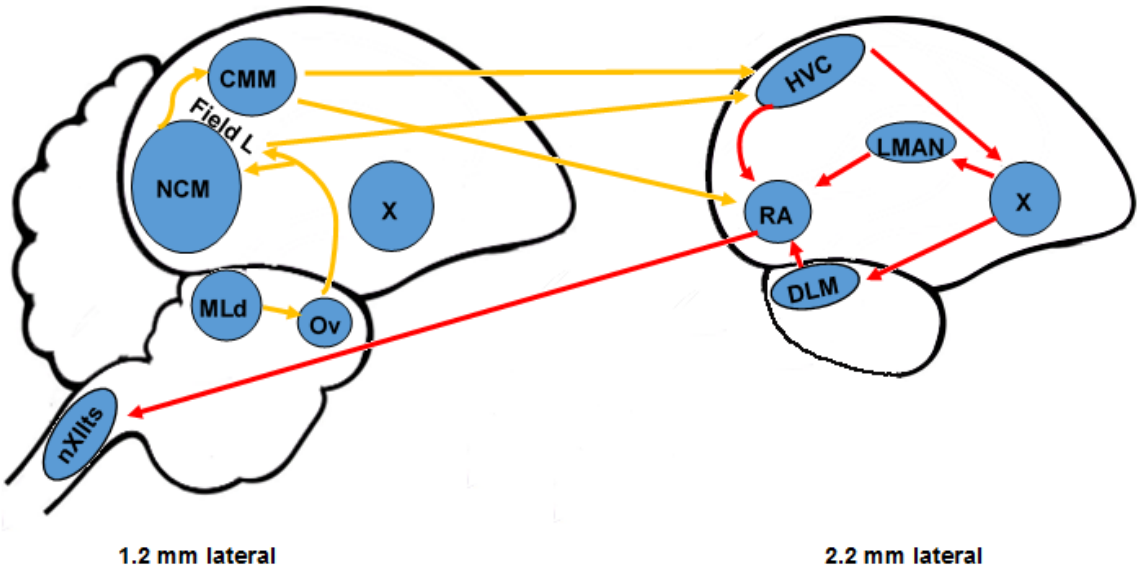

Figure 1: Auditory and Vocalization Pathway Diagram. Schematic of neural nuclei involved in the song control pathway (red arrows) and auditory pathway (orange arrows) shown on sagittal cross sections at two levels.

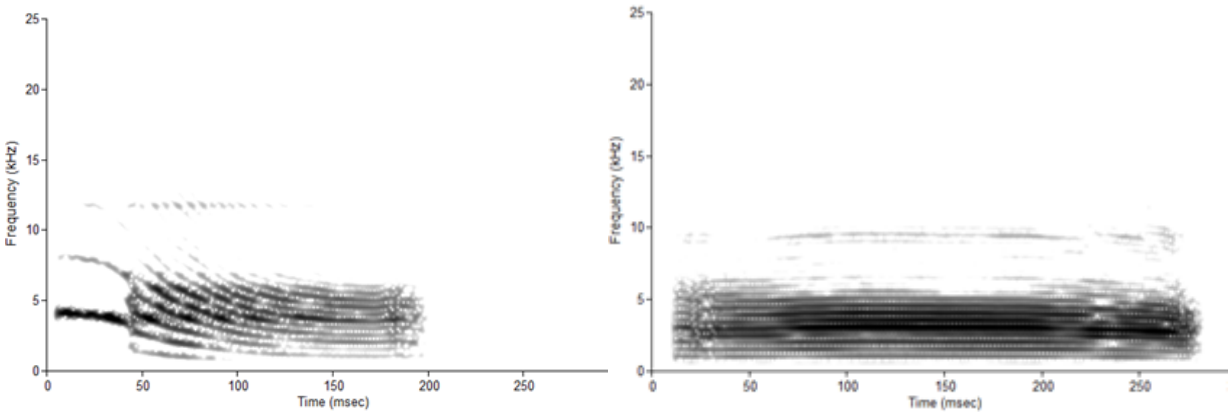

Figure 2: Comparative Sound Spectrograms. Spectrograms of male (left) and female (right) zebra finch distance calls created from stimuli used in this study.

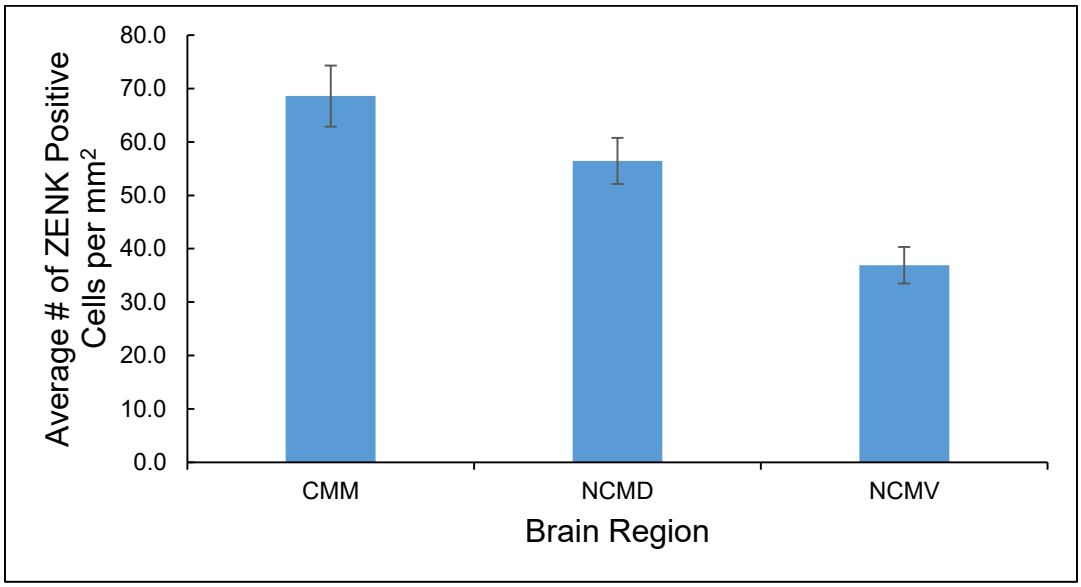

Figure 3: Average ZENK expression by brain region. The repeated measures ANOVA showed a significant main effect of Brain Region (F (2, 104) = 40.189, p<0.001) across all playback groups and both sexes. The Bonferroni-corrected pairwise comparison found a significant difference between all three regions at the p<0.001 level. Bars show mean ZENK expression with error bars representing the Standard Error of the Mean; Caudomedial mesopallium (CMM), Caudomedial nidopallium, dorsal (NCMd), and Caudomedial nidopallium, ventral (NCMv).

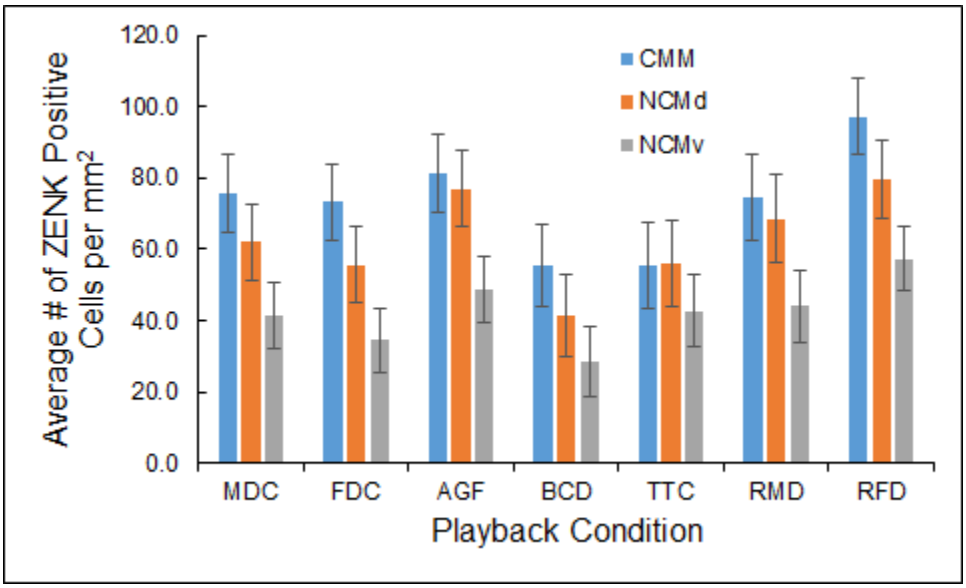

Figure 4: Average ZENK expression by brain region and playback condition. The repeated measure ANOVA showed that there was no significant interaction between brain region and playback condition. Bars show mean ZENK expression across all areas with error bars representing the Standard Error of the Mean. Playback group names and sample sizes are MDC: Male Distance Call (n=7), FDC: Female Distance Call (n=7), AGF: American Gold Finch Call (n=7), BCD: Black-capped Chickadee D-note (n=7), TTC: Tufted Titmouse Call (n=7), RMD: Reversed Male Distance Call (n=6), RFD: Reversed Female Distance Call (n=7). Brain regions are CMM: Caudomedial mesopallium, NCMd: Caudomedial nidopallium, dorsal, NCMv: Caudomedial nidopallium, ventral.

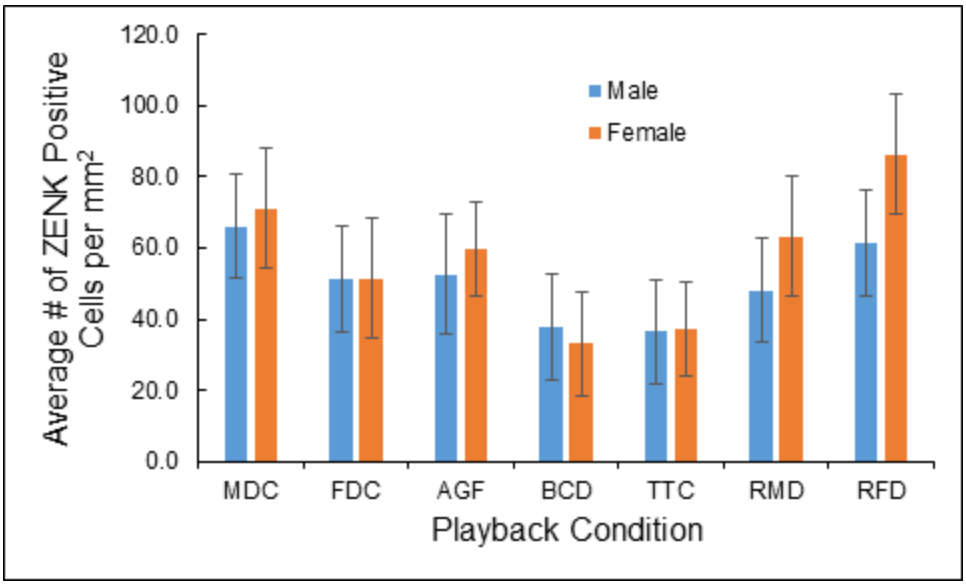

Figure 5: Average ZENK expression by playback condition and sex for zebra finches. The repeated measure ANOVA showed that there was no significant difference in Playback Condition (F (6, 312) = 0.114, p>0.05) or Sex (F (1, 52) = 0.406, p>0.05). Bars show mean ZENK expression across all areas with error bars representing the Standard Error of the Mean. 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 Gold Finch 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).

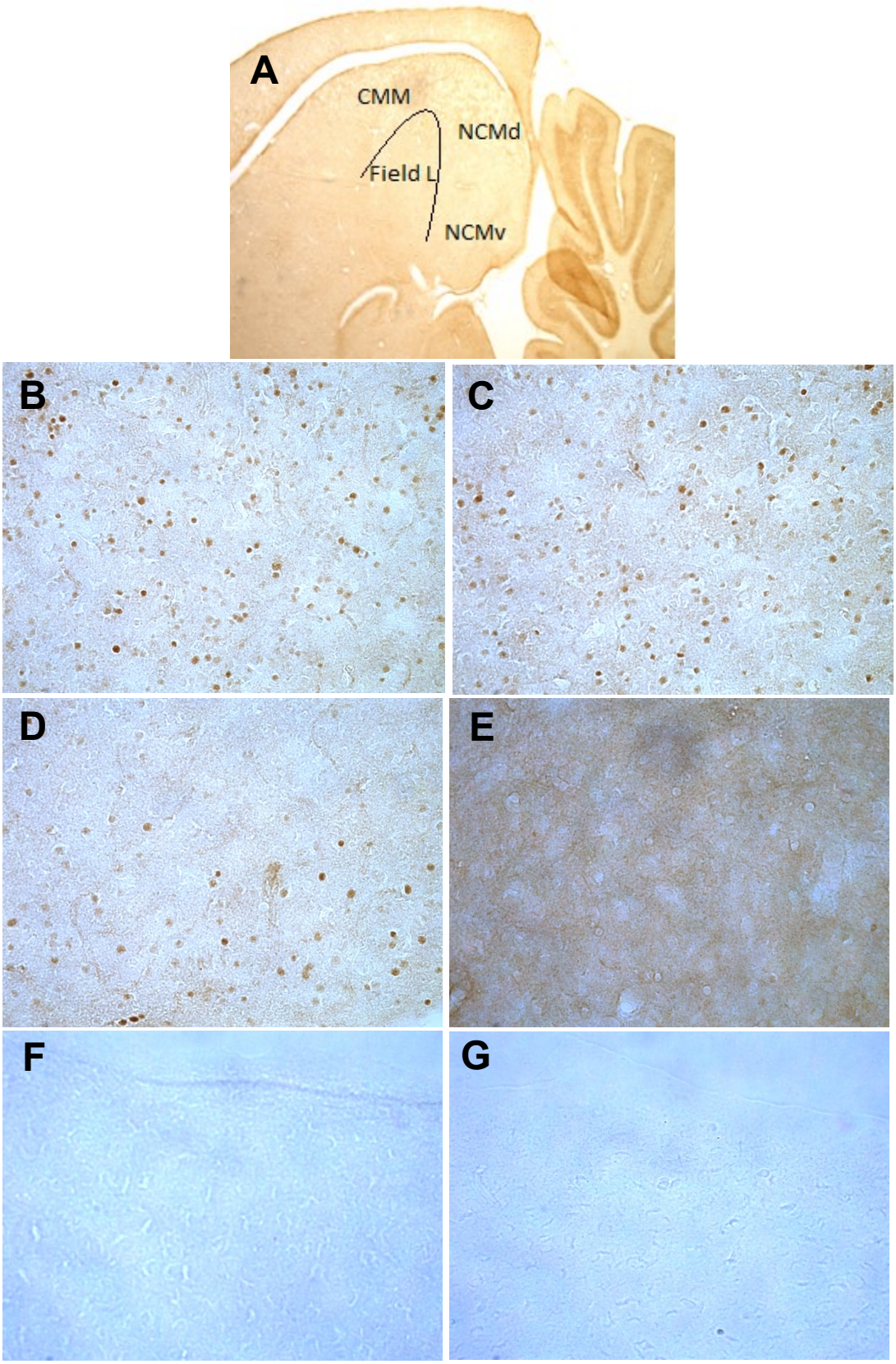
A
CMM
NCMd
Field L
NCMv
B
C
D
E
F
G

Figure 6: Example ZENK expression in auditory areas and negative controls. A) Zebra finch telencephalon at 5X magnification. Examples of ZENK expression in the three measured areas (B) Caudomedial mesopallium C) Caudomedial nidopallium, dorsal D) Caudomedial nidopallium, ventral) and negative controls (E) Field L, F) No primary, G) no secondary) taken at 40X magnification. All images taken from the same brain section from a male zebra finch in the male distance call playback group.

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