Behavioural and Neurobiological Perception of Vocal Communication in Songbirds

By

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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Abstract

Black-capped chickadees (Poecile atricapillus) are a species of North American songbirds commonly studied for their unique vocalizations. The two most studied vocalizations are their *fee-bee* song, used for mating and territory defense, and their name-sake *chick-a-dee* call, which has multiple functions including flock coordination, identifying food location, and mobbing of predators. While black-capped chickadees primarily produce *fee-bee* songs in spring, they produce *chick-a-dee* calls year-round with call production peaking in the fall. In Chapter 2 I asked if the meaning of the call may also change across seasons. For instance, flock communication could be more important in the fall than in the spring, and food type and availability change according to season. To determine if the *chick-a-dee* call varies acoustically across seasons in a predictable manner, I conducted an operant go/no-go discrimination task that examined black-capped chickadees' ability to categorize calls produced in two different seasons: fall and spring. I found that birds trained to respond to vocalizations produced in either fall or spring learned to discriminate at the same rate as birds trained to respond to pseudorandomized stimuli. I also conducted a bioacoustic analysis of the calls and found no differences in calls produced in the spring versus calls produced in the fall. These results suggest that while chickadees can be trained to discriminate between *chick-a-dee* calls produced in different seasons, they do not discriminate these calls or perceive these calls as being members of natural, preexisting, perceptual categories, based on an underlying perceptual similarity. In Chapter 3, I expanded on a previous behavioural study that asked if chickadees have a preference for duty cycles (the proportion of time filled by vocalizations). Wilson and Mennill (2011) found that chickadees show stronger behavioral responses to playback of chick-a-dee calls with higher duty cycles. That is birds responded more to higher duty cycle playback compared to lower duty cycle

playback. Here I presented chickadees with *chick-a-dee* calls with either high or low duty cycles, then measured the amount of ZENK labeled cells in the auditory nuclei. I found that there was no neurobiological difference between high and low duty cycles, differing from the previous behavioural results. Next, in Chapter 4, I conducted a methodology experiment. In large part due to its neuronal-specific labeling of ZENK protein, Santa Cruz Egr-1 sc-189 has been widely accepted as the standard primary antibody in songbird research. However, Santa Cruz Biotechnology Egr-1 no longer specifically labels and has also discontinued production of Egr-1 sc-189. Thus, Chapter 4 is focused on analyzing the effectiveness of alternative primary antibodies. Abcam monoclonal Egr-1 effectively labels ZENK in the songbird auditory nuclei, making it a suitable primary antibody replacement for Santa Cruz polyclonal Egr-1. Finally, in Chapter 5, I again aimed to replicate previous behavioural and bioacoustic studies to examine if chickadees attend to information regarding sex of the caller of the *chick-a-dee* call. Here I presented both male and female chickadees with altered *chick-a* calls (*dee* portion removed) of both sexes and measured the number of ZENK labeled cells in select auditory nuclei. I found that while there was no significant difference between male and female listeners, only calls produced by males had significantly more ZENK labeled cells than the control condition, with female produced calls not being significantly different from either of the two other groups. Overall, my thesis used multiple approaches to further understand how black-capped chickadees perceive the *chick-a-dee* calls.

Preface

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 1937).

A version of Chapter 2 of this thesis has been accepted for publication as Scully, E.N., Campbell, K.A., Congdon, J.V., & Sturdy, C.B. (Accepted November 29, 2019). Discrimination of season in black-capped chickadee (*Poecile atricapillus*) chick-a-dee calls. Manuscript submitted for publication in Animal Behaviour and Cognition. I was responsible for concept formation, data collection, data analysis, and manuscript composition. K.A Campbell and J.V. Congdon contributed to concept formation, data collection, and manuscript edits. C.B. Sturdy was the supervisory author and was involved in concept formation and manuscript revision.

A version of Chapter 3 of this thesis has been published as Scully, E.N., Schuldahaus, B.C., Congdon, C.V., Hahn, A.H., Campbell, K.A., Wilson, D.R., & Sturdy, C.B. (2019). 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. *Behavioural Brain Research*, *356*, 490-494. doi.org/10.1016/j.bbr.2018.06.006. I was responsible for concept formation, data collection, data analysis, and manuscript composition. B.C. Schuldahaus contributed to data collection and manuscript edits. C.V. Congdon, A.H. Hahn, K.A. Campbell, and D.R. Wilson contributed to concept formation and manuscript edits. C.B. Sturdy was the supervisory author and was involved in concept formation and manuscript revision.

A version of Chapter 4 of this thesis has been published as Scully, E.N., Montoya Sanchez, J., Sturdy, C.B. (2019). Abcam Monoclonal Egr-1 ab133695 is an effective primary antibody

replacement for Santa Cruz sc-189 polyclonal Egr-1 in songbirds. *Heliyon*, *5*. doi:10.1016/j.heliyon.2019.e02938. I was responsible for concept formation, data collection, data analysis, and manuscript composition. J. Montoya Sanchez contributed to data collection and manuscript edits. C.B. Sturdy was the supervisory author and was involved in concept formation and manuscript revision.

A version of Chapter 5 of this thesis has been submitted for publication as Scully, E.N., Montenegro, C. & Sturdy, C.B. (Submitted January 6, 2020). Neurobiological response to playback of black-capped chickadee (*Poecile atricapillus*) *chick-a* calls across sexes. Manuscript submitted for publication in *Behavioural Brain Research*. I was responsible for concept formation, data collection, data analysis, and manuscript composition. C. Montenegro contributed to data collection and manuscript edits. C.B. Sturdy was the supervisory author and was involved in concept formation and manuscript revision.

Acknowledgements

Through this long journey, there have been many people that have helped encourage me to stick with my dream of getting my PhD. While I cannot thank everyone personally, there are a select few who I truly do not this I would have been able to do this without. Thank you to my current and past supervisory committee members: Dr. Marcia Spetch, Dr. Kimberley Mathot, and Dr. Clayton Dickson, for your support and guidance.

Especially, I would like to thank my supervisor, Dr. Christopher Sturdy. Since first allowing me to do a neuroscience rotation course in his lab, to giving me a new home after a change in supervisors, he has been there for me. His guidance and encouragement throughout my Masters and PhD have helped me believe in myself and showed me that I am capable of more than I thought. Without his support, I truly do not believe that I would have gotten to where I am today.

My family has also stood by me through all my ups and downs. Though they may not always understand everything that I do, they always have encouraged me to follow my dreams and stand up for myself. Thank you, Mom, Dad, Racheal, Grandma and Grandpa.

And finally, thank you to my fabulous lab mates for encouraging me to continue my education and research. Jenna and Kim, you have been such inspiring women to get to know and learn alongside. All of our adventures in and out of the lab are memories that I shall treasure for my whole life. I know that the three of us are unstoppable because we have each other.

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Chapter 1: General Introduction

Vocal learners are a select group of animals that are unique in that they must learn their vocalizations from tutors. These vocal learners span many orders, from Passeriformes (songbirds), Trochiliformes (hummingbirds), Cetacea (dolphins and orcas), to Psittaciformes (parrots). Songbirds, representing the largest order of avians, produce two main types of vocalizations: songs and calls. Songs are used to attract mates and defend territories, whereas calls are used to communicate the location of food and other individuals, and facilitate the mobbing of predators (Smith, 1991; Zann, 1996).

Environmental Effects on Communication

Recent behavioural studies have demonstrated the effects of a bird's environment on the structure of vocalizations produced. As songs are mainly used to attract mates, producing the optimal song is critical to birds. Female greenish warblers (*Phylloscopus trochiloides*) prefer males with larger song repertoires and longer songs. Scordato (2018) found that a higher population density leads to decreases in the length of male song. It was also found that females prefer songs produced at a further distance from the territory center, as population density was also lower (Scordato, 2018). The length of a song, or song bout, can also inform females about the quality of a potential mate. In the common rosefinch (*Carpodacus erythrinus*) yearling males, while sexually mature, have still not developed full adult plumage or song (Parapura, Mitrus, & Golawski, 2018). Older adult rosefinches produce a song bout with a longer strophe length than the young adults. This social and environmental information encoded within a male's song may have a major effect on which females attend to them.

While songs have a major effect on breeding success, calls contain essential information for an individual and the flock's survival. Within a flock, communication can vary from where there is food to aggressive confrontations, making the ability to interpret the message within a

call crucial. Java sparrows (*Lonchura oryzivora*) create two trills that sound very similar to the human ear. While these two trills sound similar they actually have different uses: an aggressive trill and an affiliative trill. Java sparrows are able to decipher these trills from each other as aggressive trills are faster, have a higher amplitude, and have a higher entropy than affiliative trills (Furutani, Mori, & Okanoya, 2018). In zebra finches (*Taeniopygia guttata*) all calls contain some information on the individual who produced the call; however, Elie and Theunissen (2018) found that contact calls have the most individual information, while aggressive calls have the least. This makes sense in that contact calls are produced at further distances than aggressive calls, which are usually produced within line of sight.

Call production also changes in response to the intended receiver. Carolina chickadees (*Poecile carolinensis*) change their call composition depending on if they are communicating with conspecifics of the same or different flock (Coppinger, Davis, & Freeberg, 2019). When communicating with a member of the same flock, Carolina chickadees will produce fewer introductory notes, more C notes, and fewer hybrid notes. As well as changing their vocalizations based on the intended receiver, songbirds will also change their behaviour depending on the sender. Dutour and colleagues (2019) found that two species of tits change their mobbing behaviour in response to the species of the caller. Great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*) are more likely to join in mobbing a predator in the fall if a heterospecific has started the mobbing calls, as opposed to the spring when birds are more likely to be recruited in response to a conspecific caller (Dutour et al., 2019). Although the identity of the caller is critical to both behavioural and vocal responses, neurobiological studies can complete the understanding of how songbirds perceive different vocalizations.

Communication and the Brain

Unlike humans and other vocal learners, young songbirds must learn their vocalizations quickly, as most will leave their parents within a year of hatching (Smith, 2019). Due to this shortened developmental phase, it allows us to more easily study how vocal learners interact with their tutors and what happens when the sensitive learning phase is manipulated. By measuring immediate early gene (IEG) expression in auditory areas, caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM), researchers are able to view how different vocalizations affect neuronal gene expression. Female zebra finches show differential neural expression in response to male songs, depending on the types of songs their tutor produced during the sensitive phase (Diez, Cui, & MacDougall-Shackleton, 2017). For instance, females who learned conspecific isolet songs produced the least amount of IEG ZENK expression, compared to females with normal tutors, in response to wild type songs. Another study investigating the effects of early song exposure on female zebra finches found that there was an increase of ZENK expression in the caudal ventral tegmental area (VTA) in response to courtship songs, but only in females who heard adult songs during development (Barr & Woolley, 2018). The results of these studies suggest that early life experience may have a major effect on female song preference.

Neurobiological studies in songbirds can also tell us about how the brain develops both as young and as adults. When measuring IEG expression via *in-situ* hybridization, zebra finch embryos and nestlings showed a strong preference for conspecific songs (Rivera et al., 2018). Similar to results found in adult songbirds, embryos and nestlings showed a preference for conspecific vocalizations over heterospecific, and heterospecific over silence (Avey et al., 2014; Scully et al., 2017). While the majority of vocal control development occurs during the sensitive period, there is still some neurogenesis as adults (Pytte, 2016). Looking at both zebra finches and

Bengalese finches (*Lonchura striata domestica*), Polomova and colleagues (2019) found that highly variable songs produced more newborn neurons in the song system nucleus HVC (proper name) than less variable songs. Bengalese finches were also found to have more neurogenesis in the forebrain than zebra finches overall. Tracing afferent and efferent projections from the HVC has also shed more light on how songbirds learn vocalizations. While female zebra finches do not produce a song and have smaller song system nuclei, studies have shown the importance of a conspecific tutor to female song perception and preferences (Diez et al., 2017). By tracing the projections from the HVC, Shaughnessy and colleagues (2018) found that female tracts are similar to the tracts of males; they also replicated the results of other studies that found while the song system nuclei were smaller in females than males, the auditory areas were the same (Ball, Absil, & Balthazart, 1995; Ball, Casto, & Bernard, 1994).

To further understand what is happening in the brain during acoustic communication, it is also important to understand the hormones involved. The sex hormones testosterone and estrogen are known to regulate the production and learning of birdsong (Alward et al., 2018). While most auditory studies have focused on the auditory areas CMM and NCM, there are other areas that may also play a role in auditory perception. Locus coeruleus in the brainstem, which regulates catecholamines, has been found to have more ZENK expression to familiar than to novel songs. These results mimic those of the CMM and NCM, suggesting that catecholamines may be more important in the processing of social information in birds, rather than just norepinephrine as previously thought (Dai, Chen, & Sakata, 2018). Another potential benefit of including hormonal studies with behavioural studies would be to help explain potential discrepancies between neurobiological and behavioural studies. Again, examining the seasonal findings of mobbing behaviour that Dutour and colleagues (2019) reported, it is possible that the

hormones active during the seasons changed, leading to this behavioural seasonal effect. Dopaminergic and serotonergic response to vocalizations have been found to differ depending on the season (Rodriguez-Saltos et al., 2018), suggesting a possible explanation to Dutour and colleagues (2019) findings. The integration of behavioural and neurobiological data fills in any gaps that only one methodology may generate and creates a full picture of how acoustic communication functions in songbirds.

Songbird vocalizations are complex to produce and to perceive. To fully understand acoustic communication, it is necessary to conduct not only behavioural, but neurobiological studies of perception as well. Recent behavioural studies have shown how a bird's environment can influence the vocalizations that they produce, as well as how they react to others' vocalizations; however, the 'why' is still unknown. In the past few years, researchers have discussed how development plays an important role in females, who do not typically sing, as well as in males who do. In ecology, a trend towards the approach called 'Evo-mecho' has begun, which looks to integrate behavioural ecology and evolutionary mechanisms together further understand how animals respond to their environments (McNamara & Houston, 2009). Following this example and integrating behavioural observations and neurobiological studies, the songbird research community can strengthen.

Overall, acoustic communication is an ever-present part of the animal world. The drive to further understand not just how vocalizations are learned and produced, but also how they are perceived, by both vocal learning and non-vocal learning animals is of interest to researchers. Songbirds provide a strong study species that covers multiple habitats, thrives in a natural or laboratory setting, and is an example of a vocal learning species (Lipkind et al., 2013). By further understanding how acoustic information is processed neurally in songbirds, we may gain insight

into other vocal learners such as cetaceans, and non-vocal learners such as fishes. By combining the techniques and findings of both behavioural and neurobiological studies, the field is starting to fully understand acoustic communication.

Study Species

While there are multiple species of songbirds whose vocalizations have been studied, one of the most commonly used is the zebra finch. Native to Australia, the majority of the birds used in research have been bred in captivity, allowing for genetic and developmental manipulation (e.g., Wade & Arnold, 1996; Kruijt et al., 1983). Sexually dimorphic, only the males sing, leading to many studies examining behavioural and neuronal differences between male and female birds (e.g., Vicario et al., 2001a; Vicario et al., 2001b; Gobes et al., 2009). As laboratory animals, zebra finches have greatly expanded our understanding of the songbird brain and development; however, as more female songbird species are being found to sing (e.g., Stripe-breasted wren, *Cantorchilus thoracicus*; Northern cardinal, *Cardinalis cardinalis*; European robin, *Erithacus rubecula*), the results derived from zebra finches are not as generalizable as were once thought.

Unlike the zebra finch, black-capped chickadees do not breed in captivity and are sexually monomorphic. Female chickadees have also recently been shown to produce a song similar to that of males (Hahn et al., 2015). Black-capped chickadees are native to much of North America and do not migrate during the winter (Smith, 1991). The ready availability of this species allows for direct comparisons between behavioural laboratory research and observational field work. Black-capped chickadees have also been shown to do well in laboratory operant conditioning paradigms that require categorization of different vocalizations (Hahn et al., 2015; Hahn et al., 2016; Congdon et al., 2019).

Also unique to black-capped chickadees is their complex namesake call, the *chick-a-dee* call. While most songbird species have a simple call and complex song, the chickadee has the opposite with a simple song, the *fee-bee* song, and multiple calls, including the complex *chick-a-dee* call. The *chick-a-dee* call is comprised of four note types: A notes, B note, C notes, and D notes (Ficken et al., 1978). Previous research suggests that while the A note is innate, the other note types depend, at least somewhat, on vocal learning as is seen in songs (Hughes, Nowicki, & Lohr, 1998). Chickadees use their call year-round for multiple purposes. Produced by both sexes, the *chick-a-dee* call is used for food location, flock communication, individual identification, and predator alarm (Smith, 1991; Ficken & Witken, 1977).

While many behavioural studies have focused on how chickadees react to hearing both conspecific and heterospecific calls, there have not been as many neurobiological studies on the perception of calls. Focusing on the black-capped chickadee *chick-a-dee* call, I will explore the relationships between behavioural and neuronal reactions to hearing calls.

Current Studies

This thesis aims to further expand the field songbird neuroethology by using behavioural, bioacoustic, and neurobiological approaches to increase our understanding of songbird vocal communication perception. In Chapter 2, I use an operant go/no-go conditioning paradigm to examine if black-capped chickadees categorize *chick-a-dee* calls produced in the fall versus produced in the spring as distinctly different. In addition to the behavioural paradigm, I also conducted a bioacoustic analysis of calls produced in fall versus spring, focusing on potential differences that could explain the behavioural results. Chapter 3 expands on a previous behavioural study conducted by Wilson and Mennill (2011). Wilson and Mennill (2011) found an increase in behavioral responses to calls with high duty cycles (proportion of time filled by

vocalizations), I therefore wondered if this increase in responding would be mirrored by neural expression in auditory areas of the avian brain. Due to a novel problem in my field of research, Chapter 4 deviates slightly from purely experimental to methodological in an effort to find a replacement primary antibody used to visualize ZENK protein expression in songbirds. In Chapter 5, using the novel antibody found in Chapter 4, I examine how chickadees perceive just the *chick-a* portion of the *chick-a-dee* call at a neural level. I also explore possible sex differences in perception to same versus different sex vocalizers and examine if my results are similar to those of previous behavioural and bioacoustic studies. Finally, Chapter 6 presents a general discussion of my dissertation. Overall, my thesis aims to show the importance of multiple approaches to the same question in order to gain a complete understanding of chickadee vocalizations.

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Chapter 2: Discrimination of Season in Black-Capped Chickadee (*Poecile atricapillus*) Chick-a-dee Calls[1]

[1] *A version of this chapter has been accepted for publication. Scully, E.N., Campbell, K.A., Congdon, J.V., & Sturdy, C.B. (Accepted November 29, 2019). Discrimination of season in black-capped chickadee (Poecile atricapillus) chick-a-dee calls. Manuscript submitted for publication in Animal Behaviour and Cognition.*

Introduction

Animals employ numerous modes of communication, including auditory, visual, chemical, electrical, tactile/thermal, and vibration signals (Bradbury & Vehrencamp, 1998). Birds, like many other animals, use auditory signals to communicate both within and between species groups. Vocal learners are a unique group of animals (including humans, hummingbirds, and dolphins) that require a tutor to learn vocalizations. Songbirds (of the Oscine suborder of Passeriformes) are unique from most birds in that they are vocal learners and are capable of producing more complex vocalizations than non-songbirds due to their unique use of the vocal organ, the syrinx (Greenewalt, 1968). Songbirds produce two types of vocalizations, calls and songs, which differ in both form and function. Calls are typically acoustically simple in structure and are used for localizing food, contact between individuals, and alerting others to predators (Ficken & Witkin, 1977; Mahurin & Freeberg, 2009; Templeton, Greene, & Davis, 2005). Songs tend to be more acoustically complex than calls and are mostly reserved for mating and territory defense purposes (Ficken, Ficken, & Witkin, 1978; Smith, 1991). Unlike the calls of most songbird species, the namesake *chick-a-dee* call produced by black capped chickadees (*Poecile atricapillus*) is actually more structurally complex than the species' *fee-bee* song. The *fee-bee* song consists of two notes that are produced in a stereotyped fashion (Ficken, et al., 1978).

Using operant go/no go discrimination tasks, Hahn and colleagues (2015, 2016) have shown how black-capped chickadees perceive songs differently depending on information about the signaler. For example, when provided with male and female songs, chickadees are able to discriminate between the sex of the signaler (Hahn et al., 2015). In another experiment, when provided with songs produced by chickadees in Ontario versus chickadees in British Columbia, chickadees were able to discriminate between songs produced in different geographic regions

(Hahn et al., 2016). Thus, through these two experiments, it has been demonstrated that there are acoustic differences that vary sufficiently in the *fee-bee* song to indicate sex and the geographic region of the signaler.

In contrast to the simplicity of the chickadee *fee-bee* song, the *chick-a-dee* call consists of four note types that are produced in a fixed order (A → B→ C → D), though note composition and the overall number of notes can change (Ficken et al., 1978). This call serves a role in maintaining flock cohesion and communicating the location of food sources, as well as a warning of the presence of predators and recruiting con- and heterospecifics to mob nearby predators (Smith, 1991). With all the previous work conducted on the perception of the *fee-bee* song, the question of how chickadees perceive differences in calls remains unanswered. A recent study by Congdon and colleagues (Unpublished) showed that when provided with mobbing calls produced in response to high-threat and low-threat predators, chickadees were able to discriminate between these vocalizations based on the level of arousal contained within the call (i.e., threat posed). This study provided insight into how chickadees perceive and respond to differences in calls produced under imminent contexts (e.g., predator threat), but how chickadees respond to differences in calls produced in additional varying contexts, such as season, remains untested.

The *chick-a-dee* call is produced regardless of season by both males and females with a peak in production occurring in the fall (Figure 2.1; Avey et al., 2008; Avey et al., 2011). Due to changes in food supply and social structure across seasons, we are able to assume that the information contained in the *chick-a-dee* call might also change. In the spring, winter flocks which typically range from two to twelve adult chickadees, breakup for members to find mates and produce offspring (Smith, 1991). Changes in hormones have been shown to alter song

production and call perception in other songbirds depending on seasons, suggesting there may be a perceptual difference in chickadee *chick-a-dee* calls as well (Cynx et al., 2005; Alward et al., 2017; Rodriguez-Saltos et al., 2018). Just prior to and during the breeding season, it has been found that many songbirds have relatively low mobbing rates, suggesting that mobbing predators may increase the risk of an unsuccessful breeding (Shedd, 1983). However, in the summer, when birds are raising their young, mobbing rates are highest in response to conspecific calls. The findings of Shedd (1983) contradict those of Dutour and colleagues (2018) who found higher rates of mobbing in winter in response to heterospecific calls. Since *chick-a-dee* calls function in flock cohesion, this may be one driving force underlying why *chick-a-dee* call production peaks during fall.

Due to these potential differences in call use across seasons, we predicted that there may be corresponding differences in acoustic structure or delivery (syntax) or that black-capped chickadees could attend to in order to discriminate between *chick-a-dee* calls produced in the fall versus calls produced in the spring. Based on previous success with the operant go/no-go task in determining acoustically-distinct categories perceived by chickadees (e.g., Hahn et al., 2015; Hahn et al., 2016; Congdon et al., 2019), we conducted an operant go/no-go discrimination task using male and female *chick-a-dee* calls produced in two different seasons (fall and spring), and tested in a third (winter), to investigate if black-capped chickadees could first learn to categorize the calls by season, then generalize those categories to previously non-differentially rewarded stimuli. In order to control for responding to individuals, we tested additional calls produced in the fall by individuals that also provided calls produced in the spring, and recorded all subjects' responding to these same individual/different season calls.

Methods

Subjects

Eighteen black-capped chickadees (nine male, nine female; sex determined via DNA analysis of blood samples; Griffiths, Double, Orr, & Dawson, 1998) were caught between January and February, 2016. These subjects had previous experience on other acoustic discrimination tasks, but not in discriminating conspecific *chick-a-dee* calls. All chickadees were at least one year of age at time of capture (determined by examining the color and shape of their outer tail rectrices; Pyle, 1997) in Edmonton (North Saskatchewan River Valley, 53.53˚N, 113.53˚W, Mill Creek Ravine, 53.52˚N, 113.47˚W) and Stony Plain (53.46˚N, 114.01˚W), Alberta, Canada.

During the experiment, chickadees were housed individually in operant chambers (see **Apparatus** below) and maintained on a natural light: dark schedule for Edmonton, Alberta, Canada for the winter season (December 21, 2016 to March 20, 2017). Birds had free access to water, cuttlebone, and grit, and were given one superworm (*Zophobas morio*) twice daily as nutritional supplementation. Correct responding during the operant discrimination task was rewarded with the presentation of food (Mazuri Small Bird Maintenance Diet; Mazuri, St. Louis, MO, U.S.A.), which also acted as the birds' daily food allowance. This research was conducted with the approval of the University of Alberta Animal Care and Use Committee for Biosciences, meeting the standards of the Canadian Council on Animal Care.

Apparatus

For the duration of the experiment, chickadees were housed in individual modified Jupiter Parakeet cages (30 x 40 x 40 cm, Rolf C. Hagen, Inc., Montreal, QC, Canada) inside a ventilated, sound attenuating chamber. Each cage had a water bottle, a cuttlebone, a grit cup, and

three perches. An opening (11 x 16 cm) on the side of the cage provided each chickadee access to a motor-driven feeder (see Njegovan, Hilhorst, Ferguson, & Weisman, 1994 for feeder details). Infrared cells in the feeder and on the request perch (the perch closest to the feeder) monitored the position of the bird. A personal computer connected to an experiment controller board scheduled trials and recorded responses to stimuli. Stimuli were played from the personal computer hard drive, through either a Cambridge A300 Integrated Amplifier, Cambridge Azur 640A Integrated Amplifier (Cambridge Audio, London, England), or an NAD310 Integrated Amplifier (NAD Electronics, London, England) and through a Fostex FE108 Σ or Fostex FE108E Σ full-range speaker (Fostex Corp., Japan; frequency response range 80-18,000 Hz) located beside the feeder. See Sturdy and Weisman (2006) for a detailed description of the apparatus.

Acoustic Stimuli

A total of 250 black-capped chickadee *chick-a-dee* calls were used as stimuli in the experiment, with 140 calls produced in the fall and 110 calls produced in the spring. To avoid the possibility of an individual stimulus influencing the experiment, 22 male and female individuals provided 110 of the calls produced in fall and 21 different male and female individuals provided the 110 calls produced in spring (hereby referred to as Fall 1 and Spring). The remaining 30 calls produced in the fall were provided by six individuals that had also provided spring-produced calls; however, these fall-produced calls were used only in a transfer stage to investigate possible individual effects (i.e., Fall 2). All stimuli were bandpass filtered (400 Hz-13,000 Hz; outside the frequency range of each vocalization type) using GoldWave version 5.58 (GoldWave, Inc., St. John's, NL, Canada) to reduce any background noise. For each stimulus, 5 ms of silence was added to the leading and trailing portion of the vocalization. The first 5 ms of the vocalizations

were tapered to remove transients, then the amplitude was equalized using SIGNAL 5.10.24 software (Engineering Design, Berkeley, CA, USA). Stimuli were presented at approximately 75 dB as measured by a Brüel & Kjær Type 2239 (Brüel & Kjær Sound & Vibration Measurement A/S, Nærum, Denmark; A-weighting, slow response) decibel meter at the approximate height and position of a chickadee's head when on the request perch.

Acoustic Analysis. We measured multiple acoustic features using SIGNAL software, including the $F_{(max)}$ of the entire call, duration, number of individual note types, and the total number of notes per acoustic stimulus. We then ran multiple independent samples t-tests for each of the measures between calls produced in fall versus spring. We found that there were no significant differences between calls produced in the fall versus the spring for the following measures: $F_{(max)}$ (t_{248} = 1.373, p = 0.171, CI = -116.13 - 650.47), duration (t_{248} = -1.308, p = 0.192, CI = -240.05 - 48.45), A-notes (t_{248} = -1.350, p = 0.178, CI = -0.92 - 0.17), B-notes (t_{248} = -0.957, p = 0.339, CI = -0.51 - 0.18), C-notes (t_{248} = -1.015, p = 0.311, CI = -0.38 - 0.12), D-hybrid notes (t_{248} = 1.756, p = 0.08, CI = -0.1 - 0.16), and D-notes (t_{248} = -1.615, p = 0.108, CI = -1.11 - 0.11). However, we did find that there were significantly more notes per call in spring compared to fall calls (t_{248} = -2.881, p = 0.004, CI = -1.85 - -0.35).

Procedure

Pretraining. Once birds learned to use the request perch and feeder to obtain food, they were moved onto Pretraining. In Pretraining, birds were trained to respond to all stimuli (future S+, S-, and transfer stimuli) and received food for all responses. Each trial started with a bird landing on the request perch and remaining between 900 and 1100 ms. A randomly-selected stimulus was played without replacement until all 250 stimuli had been heard. If the bird entered the feeder within the 1 sec interval after the stimulus stopped playing, it was given access to food for 1 sec,

followed by a 30s intertrial interval with the houselight on. If the bird left the request perch before the entire stimulus played, the houselight would turn off for a 30s timeout, and the trial would be considered interrupted. If the bird sat on the perch for the full length of the stimulus, but then did not move off the perch for the 1 sec after, a 60s intertrial interval was started; however, the intertrial interval was terminated if the bird left the perch.

Birds were required to stay on pretraining until they had completed six 500-trial blocks of ≥ 60% responding on average to all stimuli, at least four 500-trial blocks ≤ 3% difference in responding to future S+ and S- stimuli, and at least four 500-trial blocks ≤ 3% difference in responding to each transfer stimuli type (Fall 1, Spring, Fall 2). Once criteria was reached, each bird was given a day of free feed, followed by a second round of pretraining where they only needed one 500-trial block of ≥ 60% responding on average to all stimuli, one 500-trial block ≤ 3% difference in responding to future S+ and S- stimuli, and one 500-trial block ≤ 3% difference in responding to each transfer stimuli type (Fall 1, Spring, Fall 2).

Discrimination Training. The procedure was the same as during Pretraining; however, only the 120 training vocalizations were presented (with the remaining 130 withheld for use during transfer testing), and responding to half of these vocalizations (S-) were then punished with a 30 sec intertrial interval with the houselight off. Responses to rewarded (S+) vocalizations resulted in 1 sec access to food. Discrimination training continued until birds completed six 480-trial blocks with a discrimination ratio (DR) ≥ 0.80 with the last two blocks being consecutive. For DR calculations see response measures, below.

Birds were randomly assigned to either a True category discrimination group (n = 12) or a Pseudo category discrimination group (n = 6). Black-capped chickadees in the True category discrimination group were divided into two subgroups: one subgroup discriminated 60 rewarded

(S+) calls produced in fall from 60 unrewarded (S-) calls produced in spring (Fall S+ Group; three males and three female subject), while the other subgroup discriminated 60 rewarded (S+) calls produced in spring from 60 unrewarded (S-) calls produced in fall (Spring S+ Group; three males and three female subject).

The Pseudo category discrimination group was also divided into two subgroups. Each subgroup discriminated 30 randomly-selected rewarded (S+) calls produced in fall and 30 randomly-selected rewarded (S+) calls produced in spring from 30 unrewarded (S-) calls produced in fall and 30 unrewarded (S-) calls produced in spring (Total of 120 stimuli; Pseudo 1: two males and one female subject; Pseudo 2: two males and one female subject). The purpose of the Pseudo groups was to include a control in which subjects were not trained to categorize according to season.

Discrimination 85%. This phase was identical to Discrimination training, except that the S+ vocalizations were rewarded with a reduced probability (i.e., $P = 0.85$). On unrewarded S+ trials, entering the feeder after the stimulus finished playing resulted in a 30 sec intertrial interval, during which the houselight remained on, but there was no access to food. Discrimination 85 training was employed to introduce birds to trials in which there was no access to food, but the houselight remained illuminated, in order to prepare birds for transfer trials in which stimuli were neither rewarded, nor punished. Discrimination 85 training continued until birds completed two 480-trial blocks with a DR ≥ 0.80.

Transfer Testing. During Transfer testing, the stimuli and reinforcement contingencies from Discrimination 85 were maintained. In addition, 130 stimuli (50 Fall produced calls from novel individuals, i.e., TRS Fall 1; 50 Spring produced calls, i.e., TRS Spring; and 30 Fall produced calls from repeated individuals, i.e., TRS Fall 2) were introduced. These new (i.e., transfer)

stimuli were each presented once during a 730-trial block (S+ and S- stimuli from Discrimination 85 training were presented five times each; randomly-selected without replacement). Responses to transfer stimuli resulted in a 30 sec intertrial interval with the houselight on, but no access to food; we did not differentially reinforce or punish transfer stimuli, and only presented each transfer stimulus once each per bin, so subjects did not learn specific contingencies associated with responding to these transfer stimuli. All birds completed a minimum of three blocks of Transfer trials and these were included for analysis. Following Transfer, birds were returned to their colony rooms.

One bird completed all training and testing, but was found to be a statistical outlier in transfer testing as its bins to criterion exceeded three standard deviations from the group mean. This bird's data were removed from the data set for the statistical analysis of the transfer data.

Response Measures. For each stimulus exemplar, a proportion of response was calculated by the following formula: R+/(N-I), where R+ is the number of trials in which the bird went to the feeder, N is the total number of trials, and I is the number of interrupted trials in which the bird left the perch before the entire stimulus played. For Discrimination and Discrimination 85 training, we calculated a discrimination ratio (DR), by dividing the mean proportion of response to all S+ stimuli by the mean proportion of response of S+ stimuli plus the mean proportion of response of S- stimuli. A DR of 0.50 indicates equal responding to rewarded (S+) and unrewarded (S-) stimuli, whereas a DR of 1.00 indicates perfect discrimination. The DR was used as a criterion to move to the next stage, but not a measure used for analysis.

Statistical Analysis.

An independent-sample *t*-tests was run on the number of trials to criterion and DRs for the True and Pseudo category groups. We then ran a repeated measures ANOVA on the

proportion of response to different stimulus types (DIS S+ and TRS S+; DIS S- and TRS S-) for both True and Pseudo groups. We also ran a repeated measures ANOVA on the proportion of response between transfer type (TRS Fall 1; TRS Fall 2).

Results

Trials to Criterion

We conducted an independent-samples *t*-test on the number of trials to reach criterion for the two True category conditions (S+ Fall group, S+ Spring group) to determine if there were any differences in acquisition speed. There was no significant difference found, $t_{10} = 1.061$, $p = 0.314$, $CI = -15.220 - 42.887$. We also did not find a significant difference between males and females, $t_{10} = -0.579$, $p = 0.088$, $CI = -37.977 - 22.311$.

We also conducted an independent-samples *t*-test on the number of trials to reach criterion for the two Pseudo category conditions (Pseudo 1 group, Pseudo 2 group) to determine if there were any differences in acquisition speed. There was no significant difference found, $t_4 = 0.505$, $p = 0.640$, CI = -42.020 - 60.687.

To compare the acquisition performance of the True and Pseudo category groups and to determine if True groups learned the discrimination in fewer trials than Pseudo groups, we conducted an independent-samples *t*-test on the number of 120-trial blocks to reach criterion for the True category and Pseudo category groups. There was no significant difference between the groups ($t_{16} = -1.166$, $p = 0.261$, CI = -36.407-10.574) in that True birds did not learn to discriminate significantly faster than Pseudo birds (Figure 2.2). We also did not find a significant difference between males and females, $t_{16} = 0.201$, $p = 0.660$, $CI = -20.982 - 25.383$.

Category Learning

A one-way ANOVA, excluding the outlier data, on the proportion of responding to the four stimulus types [Discrimination (DIS) S+ stimuli, Discrimination S- stimuli, Transfer (TRS) S+ associated stimuli, Transfer S- associated stimuli; excluding the TRS Fall 2 stimuli] by birds in the True Group did not find significance between season groups (Figure 2.3; $F_{1,9} = 4.552$, $p = 0.062$). A one-way ANOVA on the proportion of responding to the four stimulus types [Discrimination (DIS) S+ stimuli, Discrimination S- stimuli, Transfer (TRS) S+ associated stimuli, Transfer S- stimuli; excluding the TRS Fall 2 stimuli] by birds in the Pseudo Group did not find significance between groups ($F_{1,4} = 4.147$, $p = 0.111$).

Including the outlier data did not alter the results, with an ANOVA on the proportion of responding to the four stimulus types [Discrimination (DIS) S+ stimuli, Discrimination S- stimuli, Transfer (TRS) S+ associated stimuli, Transfer S- associated stimuli; excluding the TRS Fall 2 stimuli] by birds in the True Group did not find significance between groups ($F_{1,10} = 1.438$, $p = 0.258$).

Individual Learning

In order to determine if chickadees would respond differentially to calls produced by the same individual in different seasons, using the learned category (S+), we conducted a paired-samples *t*-test. The *t*-test compared responding to fall produced calls (Fall 2 stimuli), from individuals who also provided spring produced calls during the Discrimination Training phase, in both the S+ fall group and the S+ spring group. We did not find significance between groups ($t_{24} = 1.741$, $p = 0.094$, $CI = -0.0136 - 0.161$). This suggests that the birds did not attend more to individual than season, and treated all transfer stimuli the same.

Discussion

We found that while black-capped chickadees can learn to discriminate between the two groups of vocalizations, *chick-a-dee* calls produced in the fall versus spring; however, chickadees do not seem to naturally categorize these calls into "fall" and "spring" produced call categories. That is to say that there is nothing perceptually similar about the vocalizations produced in either the spring or fall that made them form spring-like or fall-like perceptual categories. We found no significant differences in the time to acquisition between the True and Pseudo groups (Figure 2.2), suggesting that birds learning a true category had no advantage over birds learning without a perceptual category. Additionally, we found no significant differences in responding to untrained calls, meaning there was no evidence of transfer of the categories learned in Discrimination (Figure 2.3). We also found no significant differences between responding to calls from the same individual and responding to separate individuals.

The lack of significant differences in acquisition between the True and Pseudo groups suggest that the black-capped chickadees used rote memorization, rather than categorization, to learn the task. The use of rote memorization as a mechanism to solve this task can also be supported by the fact that chickadees in the current study were unable to generalize their initial learning to novel stimuli. It is possible that using a larger stimulus set would cause the chickadees to rely on categorization rather than memorization (McGregor & Avery, 1986); however, categorization by black-capped chickadees has been observed in similar behavioural tasks with smaller conspecific-produced stimulus sets (see Hahn et al., 2016) validating our methodology for testing categorization.

While little is known about whether or how the bioacoustics of *chick-a-dee* calls change across seasons, behaviourally chickadees have been observed to change their behaviour in response to these calls depending on season. Individual Carolina chickadees (*Poecile*

carolinensis) attend to syntax, or note order, more in the fall and winter compared to the spring, likely due to the formation of flocks in the fall and winter with both conspecifics and heterospecifics (Clucas et al., 2004). As the syntax of the calls used in the current study was not manipulated in the stimuli used as it was in the Carolina chickadees study, it is likely that this effect was not present in the current study. Cells in the cochlea and brainstem of passerines have been shown to respond to sound differently depending on season (Lucas et al., 2007). Carolina chickadees, tufted titmice (*Baeolophus bicolor*), and nuthatches all showed a seasonal effect on their neural responding to differences in frequencies and onset amplitude. Rodriguez-Saltos and colleagues (2018) demonstrated that hormones produced in response to calls were seasonally modulated in white-throated sparrows (*Zonotrichia albicollis*), an effect that would not have been seen in the current study as we only collected behavioural data. These changes across seasons suggest that perhaps black-capped chickadees also change which components of the *chick-a-dee* call they attend to dependent on season, rather than a bioacoustic change in the calls. Future studies could focus on how chickadee respond behaviourally and hormonally to certain note types across seasons.

Since we did not find that black-capped chickadees naturally categorize between calls produced in the fall versus spring during Transfer testing, it seems sensible to inquire if the calls differ acoustically, as a lack of acoustical differences would be consistent with a failure to categorize calls into perceptual categories. Although we did conduct a general bioacoustic analysis of the calls used in this study, we did not look at acoustic differences in the note types specifically which could aid in learning these individual calls. Using solely the calls used in this study, our bioacoustic analysis did not find any differences between calls produced in the fall versus the spring, with the exception of number of total notes. With the lack of acoustic

differences across seasons it suggests that the chickadees use pure memorization during the training phases to learn. The lack of physical differences in calls would also explain why there was no generalization of the learned categories to the unlearned stimuli during the testing phase. Although we did conduct a general bioacoustic analysis of the calls used in this study, we did not look at acoustic differences in the note types specifically which could aid in learning these individual calls. An unpublished study conducted following the completion of this experiment aimed to quantify bioacoustic differences between individual's calls across seasons (Campbell et al., unpublished) and demonstrated changes in the composition of calls produced by individual birds in spring as compared to the calls they produced in the fall (Figure 2.1). However, these individual differences were not observed to be consistent between birds such that the changes in composition observed in one bird were often different than that of another (i.e., some birds produced more A notes in spring than they did in fall, whereas others produced fewer A notes). While the unpublished study did not find consistent differences between individuals, it is possible that with a larger sample size a species typical seasonal difference in call structure could be found. Future studies should explore more fully the bioacoustic differences across seasons as species overall. In our study, we aimed to examine if individual calls differed with season by presenting previously non-differentially rewarded calls produced by the same individuals as a trained call, but recorded in a different season. We found that chickadees responded the same to these novel calls as to those produced by novel individuals, suggesting that the chickadees were not responding to an individual any more than they were responding to season.

Conclusion

Overall, we found that black-capped chickadees do not categorize *chick-a-dee* calls produced in different seasons as distinct. While previous studies have shown behavioural,

physiological, and hormonal differences in response to *chick-a-dee* calls across season, this experiment suggests that the calls themselves do not differ between spring and fall. Although we were able to successfully train chickadees to respond depending on season a call was produced, they did not generalize their training to novel stimuli, suggesting that seasonal information is not a natural factor attended to by these birds. In addition, calls produced by the same individual in a different season were treated similarly to those produced by other birds, regardless of the season calls were produced in. Thus, our findings provide evidence that black-capped chickadee *chick-a-dee* call production does not change between the seasons of spring and fall.

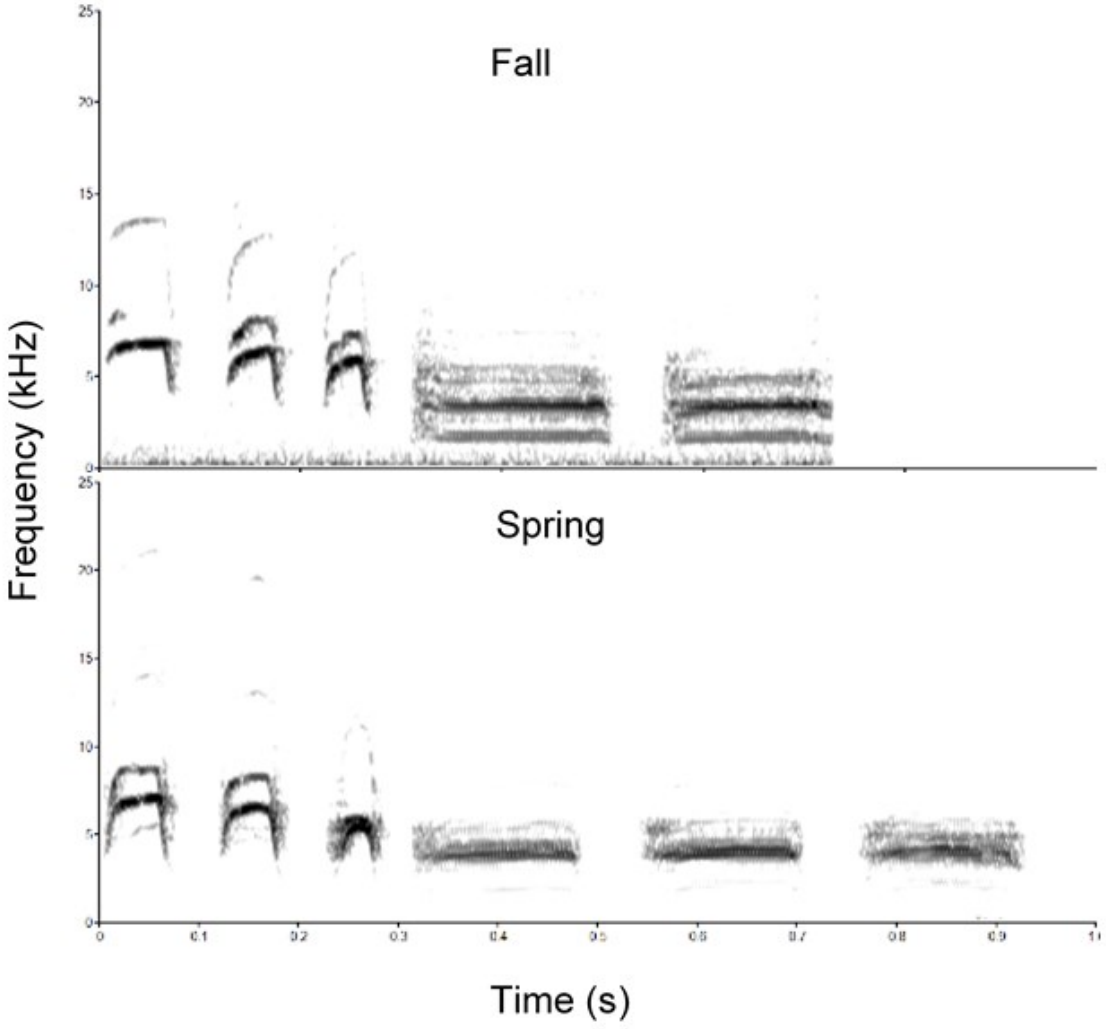

Figure 2.1. Spectrograms of calls produced in fall and spring. Male black-capped chickadee *chick-a-dee* calls produced by two different individuals. Total duration is 1 second.

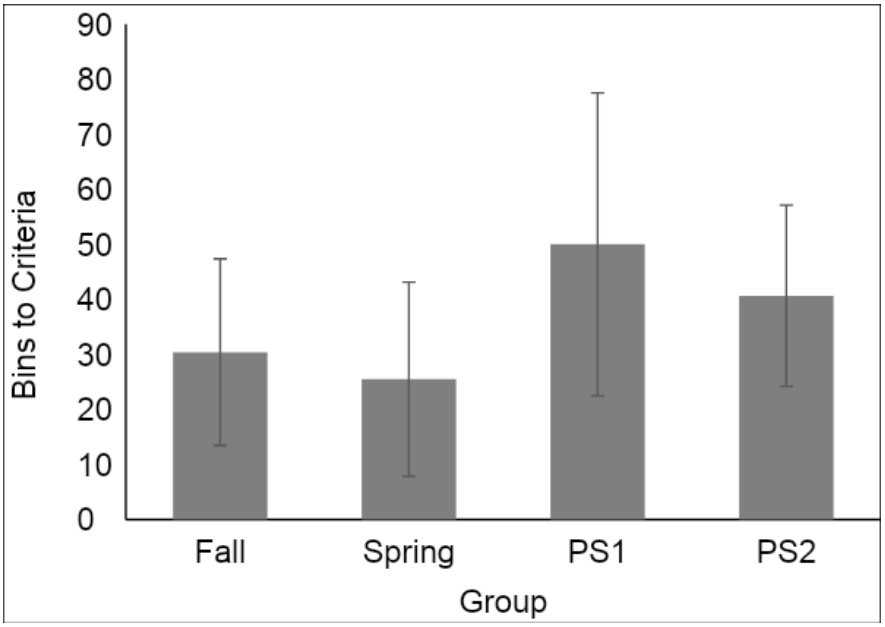

Figure 2.2. Number of bins needed to reach criteria during the DIS stage across all groups. Error bars are standard error of the mean (SEM).

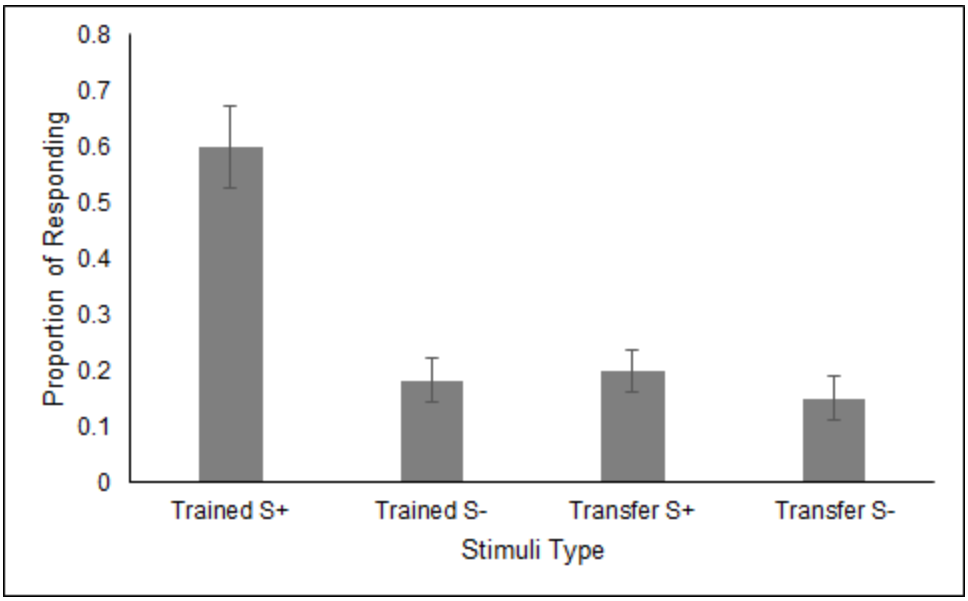

Figure 2.3. True Group proportion of responding to Trained and Transfer stimuli. Error bars are standard error of the mean (SEM).

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Chapter 3: 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[1]

[1] *A version of this chapter has been published through Elsevier. Scully, E.N., Schuldahaus, B.C., Congdon, C.V., Hahn, A.H., Campbell, K.A., Wilson, D.R., & Sturdy, C.B. (2019). 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. Behavioural Brain Research, 356, 490-494. doi.org/10.1016/j.bbr.2018.06.006.*

Introduction

Songbirds' unique use of the vocal organ, the syrinx, allows them to communicate with individuals of both their own and other species using vocalizations of varying complexity (Gill, 2007). *Chick-a-dee* calls, produced by black-capped chickadees (*Poecile atricapillus*), are used to convey a variety of information such as threat from predators (Templeton, Greene, & Davis, 2005), recruitment to food sources (Mahurin & Freeberg, 2009), recruitment of conspecifics and heterospecifics to mob perched predators (Ficken & Witkin, 1977), as well as species information (Charrier & Sturdy, 2005). Due to the complexity and relative sophistication of *chick-a-dee* calls, chickadees are a popular model species used for exploring the mechanisms behind the information encoding of acoustic signals (see Wilson & Mennill, 2011).

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 figure 3.1). 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 can be heard relative to inter-note silences), and structure (e.g., note type, note frequency) of the call (Wilson & Mennill, 2011).

Previous research has examined the vocal and behavioral responses of chickadees hearing *chick-a-dee* calls of varying acoustic structure. For example, Templeton, Greene, and Davis (2005) demonstrated that, in general, black-capped chickadees produce mobbing calls containing more D notes in response to the presence of smaller, more agile, higher-threat predators (compared to larger, less agile, lower-threat predators). This suggests that number of D notes conveys the degree of threat posed by predators. In contrast, Wilson & Mennill (2011)

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 10m 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 duty cycle, not the number of D notes, is the salient feature (see Wilson & Mennill, 2011).

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 (Wilson & Mennill, 2011), 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 Avey et al., 2011). Therefore, expression of IEG such as ZENK in the auditory areas may provide insight into how receivers perceive difference 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 (Avey et al., 2011) and behavioral results (Wilson & Mennill, 2011) 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 (Figure 3.1): (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 Avey et al., 2011). 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 previous results, we predicted the highest levels of ZENK expression would be found following playback of *chick-a-dee* calls with high duty cycles (i.e., 2 D and 10 D note structure will elicit similar levels of ZENK expression).

Methods

Subjects

Twenty male black-capped chickadees caught from three sites in Edmonton, Alberta, Canada (North Saskatchewan River Valley, 53.53N, 113.53W; Mill Creek Ravine, 53.52N, 113.47W; Stony Plain, 53.46N, 114.01W) 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; Meigs, Smith, & Van Buskirk, 1983; Pyle, 1997). Post-capture, birds were housed indoors in individual Jupiter Parakeet cages (30 x 40 x 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.

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 by Wilson and Mennill (2011). 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 Wilson & Mennill, 2011 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 Figure 1). In order to avoid pseudoreplication, each bird was presented with different calls (see Kroodsma et al., 2011 for additional details).

Playback procedure and equipment

Approximately 24 hours before playback, each bird was housed in a cage (Jupiter Parakeet), with access to food and water, in individual soundproof chambers (1.7m x 0.84m x 0.58m; 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 minutes. After this 30 minutes, birds were exposed to an hour of silence in the dark and then perfused immediately to ensure maximum quantity and quality of ZENK preservation (Mello & Clayton, 1994). 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% paraformaldehyde (PFA). The brain of each black-capped chickadee was then extracted and placed in a PFA solution for 24 hours, followed by a 30% sucrose PBS solution for 48 hours. The brains were then fast frozen using isopentane and dry ice and stored at -80°C until sectioned.

Histology

Brains were sectioned sagittally from the midline, and 48 40µm sections of each hemisphere were collected and stored in PBS. In order to visualize ZENK, sections were first washed twice in 0.1 M PBS for a minimum of five minutes, transferred to a 0.5% H_2O_2 solution and incubated for 15 minutes. Incubation was followed by three 5 min washes in 0.1 M PBS. A

second incubation in 10% normal goat serum for 20 hours at room temperature followed. Sections were then transferred into the primary antibody (egr-1, catalogue # sc-189, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 24 hours at a concentration of 1: 5,000 in 0.1 M PBS with Triton X-100 (PSB/T), then washed three times in PBS/T before being incubated in 1:200 biotinylated goat-anti-rabbit antibody (Vector Labs, Burlington, ON, Canada) in PBS/T for one hour. After three more washes in PBS/T, sections were incubated in avidin-biotin horseradish peroxidase (ABC Vectastain Elite Kit; Vector Labs, Burlington, ON, Canada) for one hour, followed by three washes in 0.1M PBS. Sections were then processed with 3,3'-diaminobenzidine tetrachloride (Sigma FastDAB, D4418, Sigma-Aldrich, Santa Fe Springs, CA, USA) to visualize expression of ZENK, followed by three washes with 0.1M PBS to remove any excess visualizing agents.

Imaging

Eight sections per individual were mounted on a slide and coverslipped. 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). Overlap in the ventral and dorsal regions of the NCM was carefully avoided. ImageJ version 1.46v was then used to quantify immunopositive ZENK cells. Using the 'Analyze Particles' functions, neuron size was defined as being between 9.07-27.21 μm^2, with a circularity between 0.4-1.00.

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. We found no significant main effects of playback condition ($F(3,16)$ 1.199, $p = 0.342$; see Figure 3.2) or significant interaction of playback condition and region ($F(3,16)$ 0.393, $p = 0.760$).

Discussion

Here we examined the extent to which ZENK expression varied in the auditory brain regions of male chickadees as a function of *chick-a-dee* call composition presented as auditory playback. Specifically, we compared calls with a low or high duty cycle and many or few D notes, to determine whether duty cycle and/or number of D notes presented had an impact on the amount of ZENK expression. We predicted that calls with a high duty cycle would lead to significantly more ZENK expression compared to calls with low duty cycle, whereas calls played in reverse would result in significantly less ZENK expression compared to all other conditions. Contrary to these predictions, we observed similar ZENK expression in response to all playback types, with playback of 2 D low duty cycle and 2 D reversed high duty cycle resulting in ZENK expression not significantly different from 10 D and 2 D high duty cycle stimuli.

Overall, our results revealed no statistically significant difference in ZENK expression among any of the groups. Notably, there were no significant differences between high and low duty cycle groups. Regardless of whether birds heard playback with many or few calls per unit time (high vs. low duty cycle), the amount of ZENK expression did not vary significantly. There

was also no significant difference between playback of 2 D high duty cycle calls and 10 D high duty cycle calls, suggesting that, neurobiologicaly at least, both were treated similarly in terms of the amount of ZENK expression. 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 behaviorally birds respond less to reversed call playback (Charrier & Sturdy, 2005) and in some cases, also show less ZENK response to reversed call note playback (Avey et al., 2011). The current finding is not unprecedented, since in some cases, reversed playback of single notes does not lead to significant reductions in ZENK expression (Scully et al., 2017; Hahn et al., 2015). Our study suggests that reversed playback may not be a compelling control stimulus, particularly in neurobiological studies.

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 (2011), 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. (2011), who 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. The calls used by Avey et al. (2011) may have contained acoustic features or information not present in the edited calls used here and by Wilson and Mennill. In fact, Templeton et al. (2005) 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 (2011). 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. 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.

Future directions

There are several proposed future directions. Most notably, we plan on replicating the current study using the calls used by Avey et al. (2011) but manipulated to vary in duty cycle in a manner consistent with Wilson and Mennill (2011). We should also conduct a study using calls manipulated following Wilson and Mennill (2011), 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, but it needs to be ruled out by an experiment designed to test the possibility. 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.

Conclusion

Here we showed that differences in *chick-a-dee* call duty cycle, while leading to differential responses behaviorally in field playback studies (Wilson & Mennill, 2011), does not lead to differential ZENK immediate early gene expression. Moreover, playback of high duty cycle calls, with or without many D notes, does not result in high levels of ZENK expression, contrary to previous work by Avey et al. (2011). Resolving these discrepancies and apparent disconnect between behavior and brain will be to focus of this work in the future.

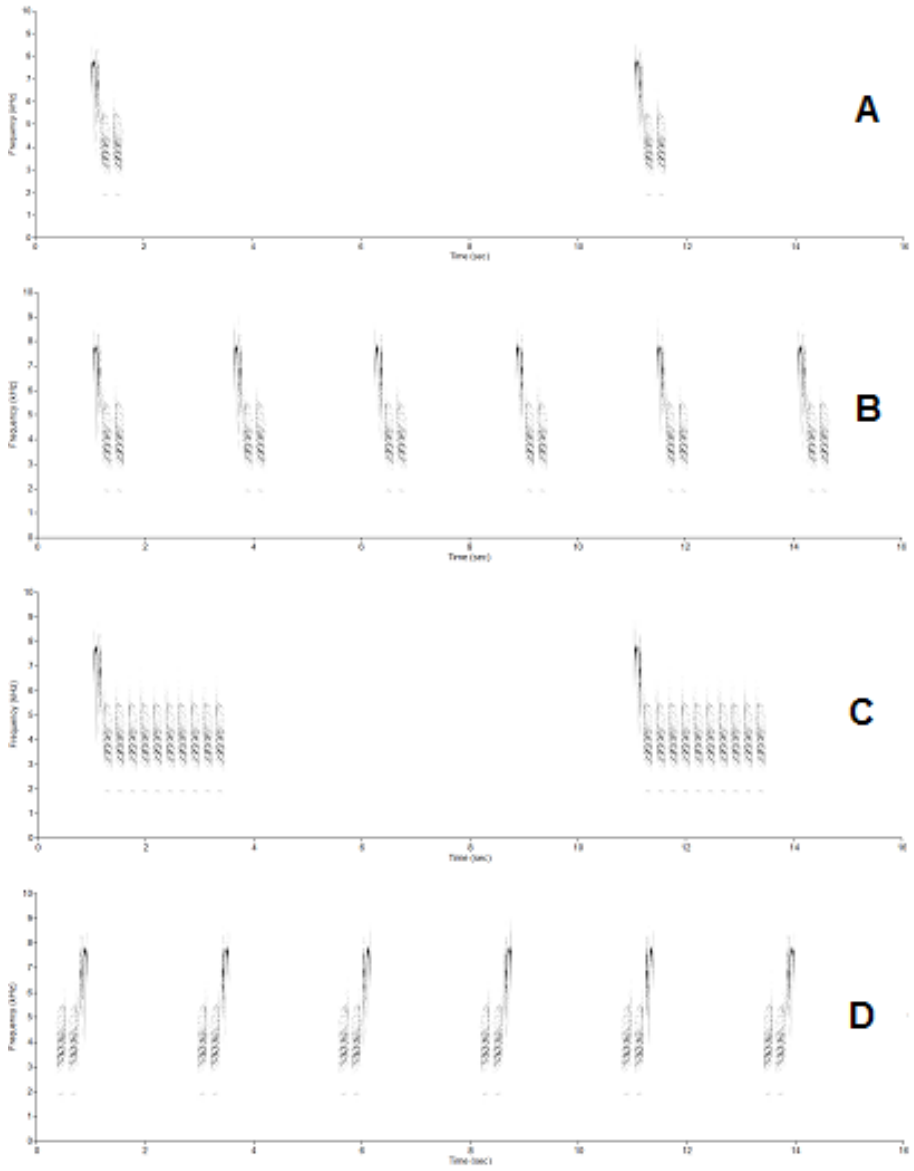

Figure 3.1: Spectrograms of Playback Stimuli. *Chick-a-dee* call with: A) 2 D notes and low duty cycle, B) 2 D notes and high duty cycle, C) 10 D notes and high duty cycle, D) 2 D notes and high duty cycle, but with the call played in reverse.

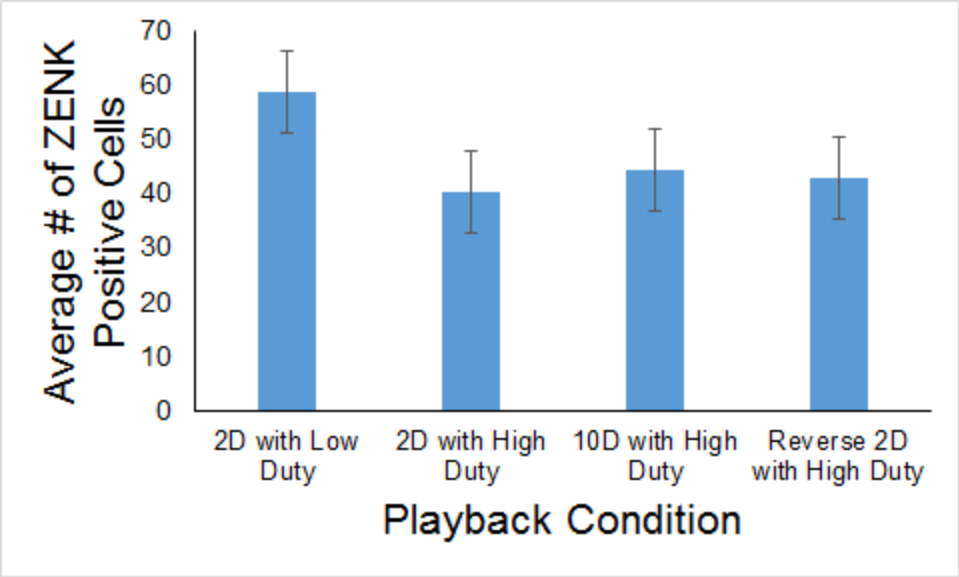

Figure 3.2: Average ZENK expression by playback condition. A repeated measure ANOVA showed that there was no significant difference in playback condition, $F(3,16)$ 1.199, p =0.342. The bar graph shows the mean ZENK expression across all areas (standardized across individuals), with error bars representing the SEM.

Acknowledgements

We thank Dan Mennill for providing playback stimuli and for his review of the manuscript.

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Chapter 4: Abcam Monoclonal Egr-1 ab133695 is an effective primary antibody replacement for Santa Cruz sc-189 polyclonal Egr-1 in songbirds[1]

[1] *A version of this chapter has been published through Elsevier. Scully, E.N., Montoya Sanchez, J., Sturdy, C.B. (2019). Abcam Monoclonal Egr-1 ab133695 is an effective primary antibody replacement for Santa Cruz sc-189 polyclonal Egr-1 in songbirds. Heliyon, 5. doi: 10.1016/j.heliyon.2019.e02938.*

Introduction

Immediate early genes (IEG) encode for transcription regulatory proteins, which have low expression when a neuron is not active. These proteins are thought to mediate long-term cellular changes involved in memory and learning (Watson & Clements, 1980; Jarvis & Nottebohm, 1997). In addition, IEGs have often been used as a tool to visualize neural activity using animal models. In songbird research specifically, the IEG protein product ZENK (zif268, Egr-1, NGFI-A, krox24) has been used to visualize how the brain responds to auditory information in the auditory nuclei caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM; Knapska & Kaczmarek, 2004). Using immunohistochemistry, and the primary antibody Egr-1, the songbird research community has made great strides in understanding where auditory stimuli are processed and how different types of auditory stimuli are responded to (see Mello et al., 1992; Jarvis & Nottebohm, 1997; Avey et al., 2011).

Unfortunately, the most widely used primary antibody (182 citations according to product website; “Egr-1 Antibody (C-19): sc-189”, n.d.) used in the field, Santa Cruz Biotechnologies Egr-1 sc-189 (Santa Cruz, CA, USA), has recently been discontinued. Also, multiple reports of un-reliable labelling of ZENK positive cells have surfaced by researchers using sc-189 produced after 2015 (unpublished observations, birdsong-l@usc.edu [Electronic mailing list]). In order to evaluate the properties of possible replacement antibodies, we tested two new primary Egr-1 antibodies, as well as one c-Fos (another type of IEG) antibody to determine whether they were effective in marking neural activity in the songbird auditory nuclei NCM, dorsal (NCMd) and ventral (NCMv), and CMM. Previous studies have shown that there is robust ZENK expression in the auditory nuclei to conspecific songs and calls (Mello & Ribeiro, 1998; Avey et al., 2014).

Following this previous research, we used conspecific songs and calls along with a silence control group, which has been shown to elicit minimal ZENK expression. In Part 1 of the experiment, we used zebra finches (*Taeniopygia guttata*) to test 11 possible protocols with novel primary antibodies, along with the Santa Cruz Egr-1 antibody as a control (12 groups total). In Part 2, we tested the generalizability of our findings by following the most successful protocol from Part 1, but using another songbird species (black-capped chickadees; *Poecile atricapillus*) as our subjects.

Methods

Subjects Part 1

Three male zebra finches of at least one year of age acquired from Eastern Bird Supplies Inc. (Thetford Mines Sud, Quebec, Canada) were used. Prior to the experimental procedure, birds were group housed in colony rooms that were kept on a 14:10 hour light:dark cycle, and maintained at 20° C. Birds were provided *ad libitum* access to food (Hagen Finch Staple VME Seed), water, and various environmental enrichment materials: perches, separators, and houses. Twice a week, birds were given a mixture of hard-boiled eggs with either spinach or parsley.

Subjects Part 2

Two adult black-capped chickadees (one male and one female; DNA analysis of blood samples confirmed sex; Griffiths, Double, Orr, & Dawson, 1998) were used. Chickadees were caught in Edmonton, Alberta, Canada (North Saskatchewan River Valley, 53.53˚N, 113.53˚W, Mill Creek Ravine, 53.52˚N, 113.47˚W) and were at least one year of age at time of capture (determined by examining the color and shape of outer tail rectrices; Meigs, Smith & Van Buskirk, 1983; Pyle, 1997). Prior to the experimental procedure, birds were housed in colony rooms were kept on the natural light:dark schedule for Edmonton, Alberta, Canada for the spring

season (March 21, 2019- June 20, 2019), and maintained at 20° C. Birds were given *ad libitum* access to food, water, and environmental enrichment materials: perches, separators, and houses. Twice a week birds were given a mixture of hard-boiled eggs with either spinach or parsley, and three times a week birds were given one superworm (*Zophobas morio*), as nutritional supplementation. This research was conducted with the approval of the University of Alberta Animal Care and Use Committee for Biosciences, meeting the standards of the Canadian Council on Animal Care.

Playback Stimuli

In Part 1, subjects were randomly assigned to hear either male zebra finch songs (n=2) or silence (n=1). In Part 2, one black-capped chickadee heard silence while the other heard male black-capped *chick-a-dee* calls. For both Part 1 and Part 2, stimuli were composed of two songs or two calls, with each call or song coming from different individual birds, played within the first 10 seconds of the stimulus, followed by 50 seconds of silence. Stimuli were created using SIGNAL software (version 5.05.02, Engineering Design, 2013) to edit the length of each stimulus and GoldWave (version 5.70; GoldWave, Inc., St. John's, NL, Canada) to bandpass filter the stimuli (350-1,300 Hz). All stimuli were presented at approximately 75 dB with a Brüel & Kjær Type 2239 sound level meter (Brüel & Kjær Sound & Vibration Measurement A/S, Nærum, Denmark; A-weighting, slow response) as measured from the middle of the playback cage.

Playback Procedure and Equipment

Approximately 24 hours before experimental playback began, each bird was singly housed in a modified cage (80 x 30 x 40 cm, Jupiter Parakeet, Rolf C. Hagen Inc., Montreal, Canada) in a sound attenuating chamber (1.7m x 0.84m x 0.58m; Industrial Acoustics

Corporation, Bronx, New York, USA), with free access to food and water. All birds were exposed to auditory playback on a loop for 30 min. To ensure maximum quantity and quality of ZENK preservation (Avey et al., 2011), birds were exposed to one hour of silence in the dark following playback, then immediately transcardially perfused. Because previous research has shown that the ZENK protein accumulates over time, we isolated the birds in the dark and silence to ensure that the ZENK protein expressed was in response to the playback (Mello & Clayton, 1994). A lethal dose of 0.04 ml of 100 mg/ml ketamine and 20 mg/ml xylazine (1:1) was administered intramuscularly. The bird was perfused via the left ventricle using heparinized 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA). The brain of each bird was then extracted and placed in a PFA solution for 24 hours, followed by a 30% sucrose PBS solution for 48 hours. Brains were fast frozen using isopentane and dry ice and stored at -80° C until sectioned. This procedure followed the standard procedure for what other ZENK songbird research has used (Avey et al., 2011; Mello et al., 1992; Park & Clayton, 2002).

Histology Part 1

Starting from the midline, 40 µm sagittal sections were collected from each brain and stored in PBS. Sections were stored in 24 well trays, with two sections per well, two trays per brain. Each tray was divided into sets of four wells that would receive the same treatment, with six groups per tray, for a total of 12 treatment groups. A) 1:1000 Santa Cruz Egr-1 sc-189 1-day incubation (Santa Cruz Biotechnology, Santa Cruz, CA, USA), B) 1:1000 Abcam Egr-1 ab133695 1-day incubation (Abcam Inc, Toronto, ON, Canada, C) 1:1000 Abcam Egr-1 ab133695 2-day incubation, D) 1:2000 Abcam Egr-1 ab133695 1-day incubation, E) 1:2000 Abcam Egr-1 ab133695 2-day incubation, F) 1:5000 Abcam Egr-1 ab133695 1-day incubation, G) 1:5000 Abcam Egr-1 ab133695 2-day incubation, H) 1:1000 Proteintech Egr-1 55117-1-AP

1-day incubation (Proteintech, Rosemount, IL, USA), I) 1:1000 Proteintech Egr-1 55117-1-AP 2-day incubation, J) 1:500 Proteintech Egr-1 55117-1-AP 1-day incubation, K) 1:1000 Abcam c-Fos ab209794 2-day incubation, L) 1:500 Abcam c-Fos ab209794 2-day incubation. All primary antibody concentrations refer to the amount of stock primary diluted in the vehicle 0.3% 0.1M PBS/Triton X-100 (i.e. 1:1000 is 1 µl of primary in 1000 µl of vehicle). Groups with a 1:1000 concentration (A, B, C, H, I, and K) were run together first, and based on preliminary results, the remaining groups (D, E, F, G, J, and L) were run at a later date with concentrations modified from the stock 1:1000 concentration depending on the preliminary results. We used a starting concentration of 1:1000 in an attempt to maintain our laboratory's protocol as closely as possible.

All sections were run using the same immunohistochemistry protocol, as follows. 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 (135 µl of 30% H_2O_2 in 7.5 ml dH_2O per tray) and incubated for 15 minutes. Three 5 min washes in 0.1 M PBS followed with an incubation in 10% normal goat serum (0.835 ml of NGS in 7.5 ml 0.3% 0.1M PBS/Triton X-100 per tray). Depending on treatment group, sections were incubated at room temperature for either 1 hour in 10% normal goat serum and 2-days in primary antibody, or incubated for 1-day in 10% normal goat serum and 1-day in primary antibody. After the incubation in normal goat serum, sections were transferred into their assigned primary antibody treatment group suspended in 0.3% 0.1 M PBS/Triton X-100 mix. The primary antibody mixture was calculated for 12 wells (4 wells × 3 trays for each treatment). In treatments with a concentration of 1:1000 (groups A, B, C, H, I, and K), 3.8µl of primary antibody was added to 3.8ml 0.3% 0.1 M PBS/Triton X-100 mix. Treatments with a concentration of 1:2000 (groups D and E) had 1.9 µl primary antibody added to 3.8 ml 0.3% 0.1

M PBS/Triton X-100 mix. Treatments with a concentration of 1:5000 (groups F and G) had 0.76 µl primary antibody added to 3.8 ml 0.3% 0.1 M PBS/Triton X-100 mix. Treatments with a concentration of 1:500 (groups J and L) had 7.6 µl primary antibody added to 3.8 ml 0.3% 0.1 M PBS/Triton X-100 mix. For each treatment, tissue in one well of a song treatment bird did not receive the primary antibody, instead incubating in 0.3% 0.1 M PBS/Triton X-100 mix as a negative control.

Sections were then washed three times in 0.1% 0.1 M PBS/Triton X-100 mix before being incubated in the secondary 1:250 biotinylated goat-anti-rabbit antibody (30 µl antibody in 7.5 ml 0.3% 0.1M PBS/Triton X-100 per tray; Vector Labs, Burlington, ON, Canada) for one hour. After three more washes in 0.1% 0.1 M PBS/Triton X-100 mix, sections were incubated for one hour in avidin-biotin horseradish peroxidase (18.75 µl 'A' and 18.75 µl 'B' in 7.5ml 0.3% 0.1 M PBS/Triton X-100 per tray; ABC Vectastain Elite Kit; Vector Labs, Burlington, ON, Canada), followed by three washes in 0.1% 0.1 M PBS/Triton X-100 mix. Sections were then processed with 3,3'-diaminobenzidine tetrachloride (1 tab of DAB plus 1 tab of UREA dissolved in 15 ml of dH_2O per 2 trays; Sigma FastDAB, D4418, Sigma-Aldrich, Santa Fe Springs, CA, USA) for 2 minutes, or until tissue was deemed too dark to visualize labeling of ZENK or c-Fos positive cells, followed by three washes with 0.1 M PBS to remove any excess visualizing agents.

Histology Part 2

Brains were sectioned sagittally from the midline, and 40 µm sections were collected and stored in PBS. Sections were stored in 24 well trays, with two sections per well, two trays per brain. All sections were run using a similar immunohistochemistry protocol as in section 2.15, but only for the treatment Group 6 (1:5000 Abcam Egr-1 ab133695 1-day incubation) with one

full tray per bird. 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 (135 µl of 30% H_2O_2 in 7.5 ml dH_2O per tray) and incubated for 15 minutes. Three 5 min washes in 0.1 M PBS followed with an incubation in 10% normal goat serum (0.835 ml of NGS in 7.5 ml 0.3% 0.1 M PBS/Triton X-100 per tray). Sections were incubated for 1-day in 10% normal goat serum and 1-day in primary 1:5000 Abcam Egr-1 ab133695 (1.52 µl primary added to 7.5 ml 0.3% 0.1 M PBS/Triton X-100 per tray).

Sections were then washed three times in 0.1% 0.1 M PBS/Triton X-100 mix before being incubated in the secondary 1:250 biotinylated goat-anti-rabbit antibody (30 µl antibody in 7.5 ml 0.3% 0.1 M PBS/Triton X-100 per tray; Vector Labs, Burlington, ON, Canada) for one hour. After three more washes in 0.1% 0.1 M PBS/Triton X-100 mix, sections were incubated in avidin-biotin horseradish peroxidase (18.75 µl 'A' and 18.75 µl 'B' in 7.5 ml 0.3% 0.1 M PBS/Triton X-100 per tray; ABC Vectastain Elite Kit; Vector Labs, Burlington, ON, Canada) for one hour, followed by three washes in 0.1% 0.1 M PBS/Triton X-100 mix. Sections were then processed with 3,3'-diaminobenzidine tetrachloride (DAB; 1 tab of DAB plus 1 tab of UREA dissolved in 15 ml of dH_2O per 2 trays; Sigma FastDAB, D4418, Sigma-Aldrich, Santa Fe Springs, CA, USA) for 1-2 minutes until desired darkness to visualize labeling of ZENK, followed by three washes with 0.1 M PBS to remove any excess visualizing agents.

Imaging

Eight sections, four per hemisphere, were mounted for each treatment separately on a microscope slide and coverslipped. Three neuroanatomical regions (CMM, NCMd, and NCMv) were subsequently imaged using a Leica microscope (DM5500B; Wetzlar, Germany) to quantify ZENK labeled cells. Four images of each region of interest were captured per hemisphere for a total of 24 images per subject. Images were obtained using a 40 x oil immersion objective lens, a

Retiga Exi camera (Qimaging, Surrey, BC, Canada), and Openlab 5.1 on a Macintosh OS X (Version 10.4.11). Overlap in the dorsal and ventral regions of the NCM was carefully avoided by imaging the dorsal most and ventral most regions. ImageJ version 1.46v was used to quantify immunopositive ZENK or c-Fos cells. Using the 'Analyze Particles' functions, neuron size was defined as being between 9.07-27.21 μm^2, with a circularity between 0.4-1.00. Counts were scaled to the highest value to view the proportion of labeled cells between the treatment groups.

Results and Discussion

Due to Santa Cruz polyclonal Egr-1 no longer being produced and recent issues with non-specific labeling in newer batches, the need to replace this antibody is critically important. Previous research (e.g., Mello et al., 1992; Avey et al., 2014) demonstrated that Santa Cruz polyclonal Egr-1 labeled more ZENK positive cells in birds exposed to conspecific song compared to birds exposed to silence; however, the current study (Group A) found the reverse (Figure 4.1M). This is not the first instance that the validity or reliability of Santa Cruz polyclonal Egr-1 has been questioned. Recently, researchers have generated multiple reports of Santa Cruz polyclonal Egr-1 produced after 2015 not reliably labelling ZENK positive cells in songbird auditory nuclei (unpublished observations).

In Part 1 of the study, we found that some of the treatment groups resulted in specific ZENK labeling in the examined auditory nuclei. Abcam monoclonal EGR-1 ab133695 at a concentration of 1:1000 (Groups B and C) was found to be too concentrated. In Groups B and C the tissue darkened too much when visualized with DAB, rendering any ZENK positive cells unidentifiable and uncountable (Figure 4.1B and 4.1C). Abcam monoclonal Egr-1 ab133695 at a concentration of 1:2000 at 1- and 2-day incubation durations (Groups D and E) resulted in successfully labeled ZENK-positive cells (Table 4.1). At a concentration of 1:5000 at 1- and 2-

day incubations (Groups F and G) Abcam monoclonal Egr-1 ab133695 was found to label ZENK positive cells in the auditory nuclei (CMM, NCMd, and NCMv) with less background staining than resulted from the same antibody when used at higher concentrations (Figure 4.1F & 4.1G). Proteintech polyclonal Egr-1 55117-1-AP was found to non-specifically label ZENK-positive cells at all concentrations and incubations due to labeling in the silent condition (Groups H, I, and J). Non-specific labeling was also identified in Field L2a, which is known not to express ZENK to song or call stimuli, blood vessels, and in the silence condition (Figure 4.2; Mello et al., 1992). Abcam c-Fos ab209794 at concentrations of 1:500 and 1:1000 with a 2-day incubation (Groups K and L; Figure 4.1K & 1L) also showed non-specific labelling in Field L2a and the silent condition. Based on these results, we concluded that Abcam monoclonal Egr-1 ab133695 used at a concentration of 1:5000 with an incubation of 1-day produced the optimal staining while maintaining the original protocol of our laboratory (Table 4.1). To confirm the reliability and generalizability of our findings, we conducted Part 2 of the experiment, using Abcam monoclonal Egr-1 ab133695 at a concentration of 1:5000 with a 1-day incubation in black-capped chickadees exposed to call playback.

In Part 2 of this study, we extended the findings of Part 1 using the protocol from Group F (1:5000 Abcam Egr-1 1 day incubation) on black-capped chickadees exposed to their own conspecific calls as the auditory stimuli. There was positive ZENK labeled cells in all three auditory areas, and no labeled cells in field L2, in the brain of the bird exposed to calls, and little to no labeling in the 3 auditory areas of the bird exposed to silence (Figure 4.3). These findings are congruent with previous findings using Santa Cruz polyclonal Egr-1 (e.g., Ribeiro et al., 1998; 1992; Avey et al., 2005; Gobes et al., 2009). Our results suggest that the use of Abcam

Monoclonal Egr-1 ab133695 is a suitable replacement for Santa Cruz polyclonal Egr-1 as a primary antibody to mark ZENK positive cells in the songbird auditory nuclei.

An important factor to consider when selecting a new antibody to use is the specificity of the potential new antibody. In the current study we ran a negative control that consisted of one well not using the primary antibody on some sections, showing that the labeling was due to the primary, and not nonspecific labelling from the secondary. While we ourselves did not run a Western Blot, Abcam, the manufacturer of the antibody, did run a Western Blot using songbird cells, showing one major band associated with >99% of the signal (Abcam Scientific). In addition, the company provided more evidence of specificity by determining the dissociation constant (Kd) for the antibody as 10^-11 (Abcam Scientific). In addition, we also had positive controls in the current study in the case of running silence groups, which has previously been shown to have no to limited ZENK labeling, as well as examination of area L2a in the experimental groups (having heard songs or calls) which is also not expected to have labeled cells (Park & Clayton, 2002; Ribeiro et al., 1998). Given this information regarding previous tests of specificity and controls run in the current study, we believe we have provided sufficient current evidence supporting the effectiveness of Abcam Monoclonal Egr-1 ab133695 to be used as a primary antibody in songbirds.

Conclusion

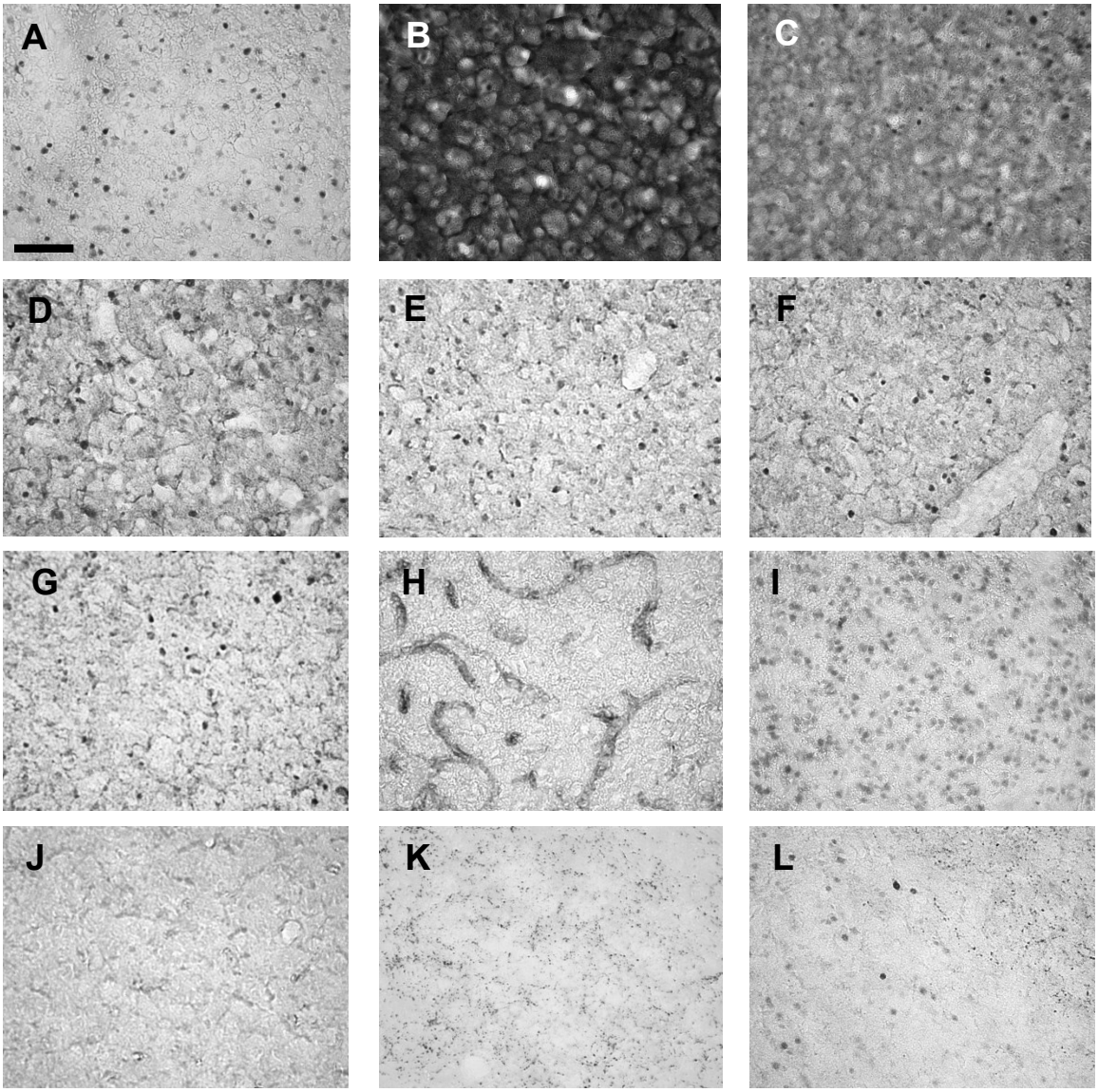
Here, we tested three new antibodies which can be used for marking IEG expression in the songbird auditory nuclei. Our results demonstrate that Abcam monoclonal Egr-1 ab133695 is a suitable primary antibody replacement for Santa Cruz polyclonal Egr-1. We showed that Abcam monoclonal Egr-1 ab133695 at concentration 1:5000 at 1-day incubation best labeled ZENK positive cells in songbirds in response to both songs and calls.

Table 4.1: Cell counts for each treatment group before and after scaling. (A) 1:1000 Santa Cruz egr-1 1-day incubation, (B) 1:1000 Abcam Egr-1 ab133695 1-day incubation, (C) 1:1000 Abcam Egr-1 2-day incubation, (D) 1:2000 Abcam Egr-1 1-day incubation, (E) 1:2000 Abcam Egr-1 2-day incubation, (F) 1:5000 Abcam Egr-1 1-day incubation, (G) 1:5000 Abcam Egr-1 2-day incubation, (H) 1:1000 Proteintech Egr-1 55117-1-AP 1-day incubation, (I) 1:1000 Proteintech Egr-1 2-day incubation, (J) 1:500 Proteintech Egr-1 1-day incubation, (K) 1:1000 Abcam c-Fos ab209794 2-day incubation, (L) 1:500 Abcam c-Fos 2-day incubation.

Treatment	*Song 1*	*Song 2*	**Average Song**	**Silence**	**Scaled Song**	**Scaled Silence**
A	26.8	20.2	23.5	42.1	0.50	0.90
B	-	-	-	-	-	-
C	-	-	-	-	-	-
D	45.0	49.3	47.2*	15.7	1.00	0.33

E	20.8	17.7	19.2	10.5	0.41	0.22
F	40.1	32.7	36.4	10.5	0.77	0.22
G	17.9	17.7	17.8	9.1	0.38	0.19
H	27.1	0	13.5	6.0	0.29	0.13
I	22.6	0	11.3	1.6	0.24	0.03
J	20.9	3.3	12.1	1.0	0.26	0.02
K	23.8	29.8	26.8	20.0	0.57	0.42
L	14.9	5.1	10.0	16.3	0.21	0.35

(*) indicates value used to scale counts


A
B
C
D
E
F
G
H
I
J
K
L

M

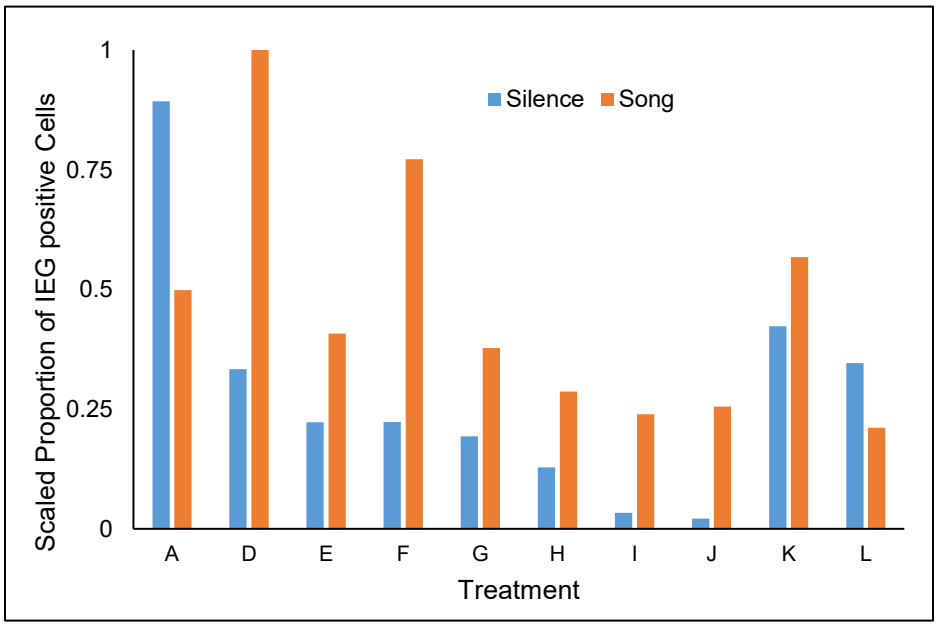

Scaled Proportion of IEG positive Cells
1
0.75
0.5
0.25
0
Silence
Song
A
D
E
F
G
H
I
J
K
L
Treatment

Figure 4.1: Effectiveness of primary antibody protocol. IEG labeling in the CMM of a song-exposed male zebra finch for each treatment; (A) 1:1000 Santa Cruz egr-1 1-day incubation, (B) 1:1000 Abcam Egr-1 ab133695 1-day incubation, (C) 1:1000 Abcam Egr-1 2-day incubation, (D) 1:2000 Abcam Egr-1 1-day incubation, (E) 1:2000 Abcam Egr-1 2-day incubation, (F) 1:5000 Abcam Egr-1 1-day incubation, (G) 1:5000 Abcam Egr-1 2-day incubation, (H) 1:1000 Proteintech Egr-1 55117-1-AP 1-day incubation, (I) 1:1000 Proteintech Egr-1 2-day incubation, (J) 1:500 Proteintech Egr-1 1-day incubation, (K) 1:1000 Abcam c-Fos ab209794 2-day incubation, (L) 1:500 Abcam c-Fos 2-day incubation. (M) Scaled proportion (scaled to the highest overall count) of IEG marked cells per treatment in silence and song-exposed zebra finch males. Counts from (B) and (C) were not included in the graph (M) as the tissue was burned during the immunohistochemistry procedure rendering them unscorable. Scale bar = 50 μm, same for all images.

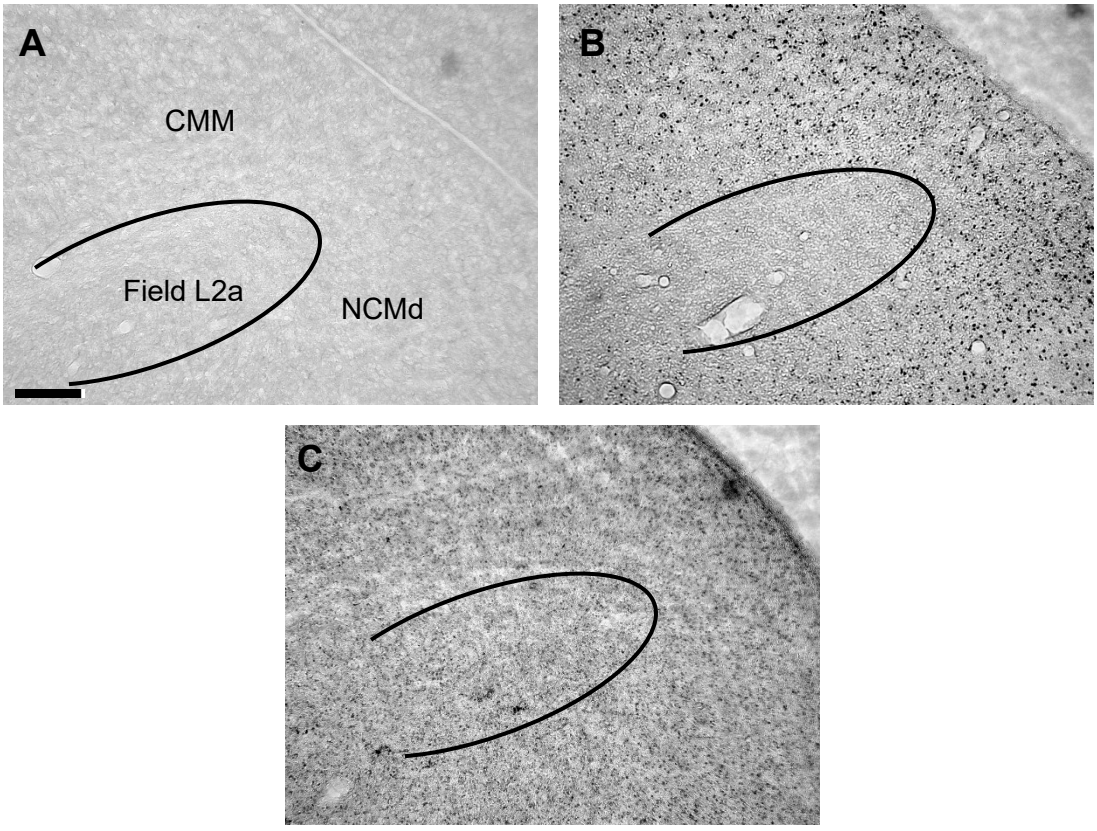


Figure 4.2: Examples of no labeling, specific labeling, and non-specific labeling. IEG labeled cells in the CMM, NCMd, and Field L2a at a 10X magnification. A) No labeling in any area in a bird who heard silence and treated with 1:5000 Abcam Egr-1 1 day incubation. B) Specific labeling in CMM and NCMd, with no labeling in Field L2a as expected, in a bird who heard songs and treated with 1:5000 Abcam Egr-1 1 day incubation. C) Non-specific labeling in Field L2a, in a bird who heard songs and treated with 1:1000 Proteintech Egr-1 2 day incubation. Scale bar =160µm, same for all images.

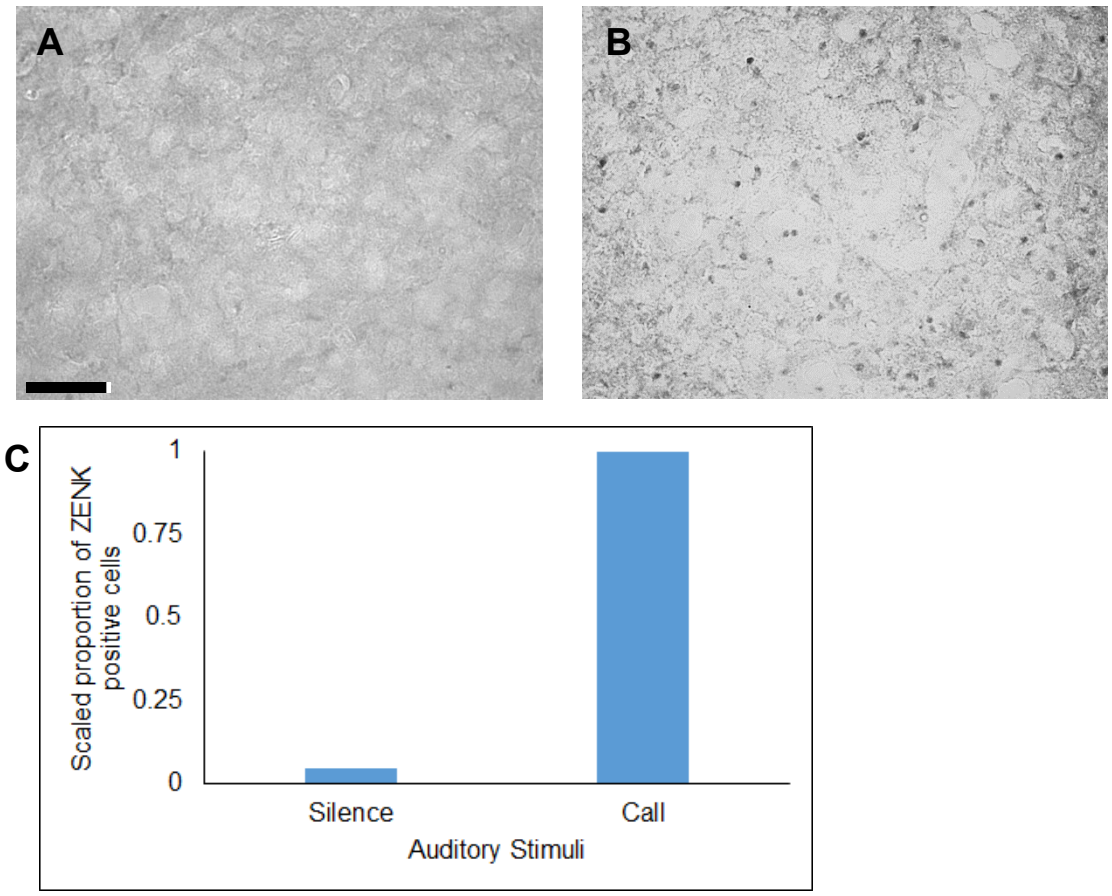


Figure 4.3: ZENK positive cells in response to call in black-capped chickadees. ZENK labeling in the CMM of the subject in the (A) silence condition and (B) conspecific call condition. (C) Scaled proportion (all counts scaled to highest count) of ZENK positive cells across all three auditory areas CMM, NCMd, and NCMv using Abcam monoclonal Egr-1 ab133695 primary antibody. Scale bar = 50 µm, same for all images.

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Chapter 5: Neurobiological response to playback of black-capped chickadee (Poecile atricapillus) *chick-a* calls across sexes[1]

[1] *A version of this chapter has been submitted for publication through Elsevier. Scully, E.N., Montenegro, C.,, & Sturdy, C.B. (Submitted February 6, 2020). Neurobiological response to playback of black-capped chickadee (Poecile atricapillus) chick-a calls across sexes. Submitted for publication in Behavioural Brain Research.*

Introduction

In most songbird species, songs are acoustically complex and used for mate attraction and territory defense. Calls tend to be simpler acoustically and are produced for multiple reasons including food location, predator alarm, and individual recognition (Catchpole & Slater, 2008). Like most songbirds, the black-capped chickadee (*Poecile atricapillus*) relies on vocal communication. The black-capped chickadee is a small North American songbird known for producing two main types of vocalizations, the *fee-bee* song and the *chick-a-dee* call (Smith, 1991). Unlike the typical songs and calls of other songbirds, the *fee-bee* song is acoustically simple two note song while the *chick-a-dee* call is more complex, consisting of four note-types.

Chickadees have been shown to change the composition of their *chick-a-dee* call depending on the situation (Mammen & Nowicki, 1981; Templeton et al., 2005). While composition of the call can change, the four note types always occur in a fixed order (A→B→C→D), with note types being repeated or omitted (Ficken, Ficken, & Witkin, 1978). For example, when recruiting other birds to mob a predator, chickadees increase the number of D notes to represent the level of threat (Templeton et al., 2005).

Many studies, both behavioural and neurobiological, have focused on the importance *dee* portion of the *chick-a-dee* call (made up of just the D notes; e.g., Dawson et al., 2006; Bloomfield, Farrel, & Sturdy, 2008; Avey et al., 2014). Expanding on the work of Templeton and colleagues (2005) measuring vocalizations in response to visual predators, Avey and colleagues (2011) conducted a neurobiological study measuring ZENK expression in response to both predator calls and mobbing calls made in response to predators of differing threat levels. They found that mobbing calls and predator calls of the same threat level resulted in similar ZENK levels, and greater ZENK labeling with high threat over low threat (Avey et al., 2011).

This is just one example of how behavioural and neurobiological studies have complemented each other.

Here, we will be focusing on the *chick-a* portion (made up of A, B, and C notes) of the *chick-a-dee* call. A recent study once more expanded on the results of Templeton and colleagues (2005), Billings, Green, and Jensen (2015) found that in response to hearing high-threat predators, chickadees produced not just more *chick-a-dee* calls, but also more *chick-a* calls both during and after playback. While we do not fully understand the function of a *chick-a* call (as opposed to the *chick-a-dee* call in its entirety), these findings suggest that *chick-a* calls may play a role in predator alarm. Additionally, Campbell and colleagues (2016) conducted a bioacoustic analysis of all *chick-a-dee* call note types and found that A notes contained information regarding the caller's sex more so than B, C, or D notes, thus providing further suggestions on how chickadees may use the *chick-a* portion of their call.

However, subsequent studies investigating the role of sex identification in the *chick-a-dee* call using an operant go/no-go task, (Campbell et al., 2020) found chickadees did not categorize calls by the sex of the caller. When birds were first trained to respond to either male or female *chick-a-dee* calls, they found no difference in responding between groups or sexes. When new birds were trained using only the *chick-a* portion (i.e. with the *dee* portion removed) of the same calls, the group trained to respond to female *chick-a calls* did transfer their training to untrained female stimuli; however, there still was no effect of sex, nor was there a difference between groups in responding to untrained male stimuli (Campbell et al., 2020). These findings suggest that while the A note contains information that differs depending on the sex of the caller, birds are not using this information, at least in the context of solving an operant discrimination task.

Due to the findings of Campbell and colleagues (2020), we questioned whether there would be any neurobiological differences in response to the *chick-a* portion of the black-capped chickadee *chick-a-dee* call in males and female black-capped chickadees. We conducted a playback experiment using both male and female produced *chick-a* calls and measured the number of ZENK positive cells in three auditory areas to visualize neural reactivity in order to identify any possible sex differences in perception. We predicted that there would be a difference in how male and female chickadees responded neurobiologicaly to same or different sex calls, as supported by the bioacoustic findings of sex differences in the A notes (Campbell et al., 2016).

Methods

Subjects

Fourteen black-capped chickadees (7 males and 7 females) caught from two sites in Edmonton, Alberta, Canada (North Saskatchewan River Valley, 53.53N, 113.53W; Mill Creek Ravine, 53.52N, 113.47W; Stony Plain) were used in this study. All birds were captured between 7 February, 2015 and 23 January, 2018, and were at least one year of age when captured. Post-capture, birds were housed indoors in individual Jupiter Parakeet cages (30 x 40 x 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 and three to five sunflower seeds daily.

Playback Stimuli

Black-capped chickadees *chick-a-dee* calls were recorded from six males and six females in individual soundproof chambers (1.7m x 0.84m x 0.58m; Industrial Acoustics Corporation, Bronx, New York, USA). One *chick-a-dee* call was randomly selected from each individual, and the *dee* portion was selected and removed using SIGNAL 5.10.24 software (Engineering Design, Berkeley, CA, USA). One *chick-a* call from each of two different individuals of the same sex were combined and separated by three seconds of silence to create one stimulus, for a total of three male and three female stimuli.

All stimuli were bandpass filtered (400 Hz-13,000 Hz) outside the frequency range of each vocalization type using GoldWave version 5.58 (GoldWave, Inc., St. John's, NL, Canada) to reduce any background noise. For each manufactured stimulus, 5 ms of silence was added to the leading and trailing portion of the vocalization. The first 5 ms of the stimuli were tapered to remove transients, then the amplitude was equalized using SIGNAL 5.10.24 software (Engineering Design, Berkeley, CA, USA). Stimuli were presented at approximately 75 dB as measured by a Brüel & Kjær Type 2239 (Brüel & Kjær Sound & Vibration Measurement A/S, Nærum, Denmark; A-weighting, slow response) decibel meter.

Playback procedure and equipment

Throughout playback, birds were kept in modified cages (Jupiter Parakeet), with free access to food and water. Birds were housed in individual soundproof chambers (1.7m x 0.84m x 0.58m; Industrial Acoustics Corporation, Bronx, New York, USA) for approximately 24 hours before playback. All birds were first exposed to 30 min of pre-playback silence, followed by 30 min of playback. Post-playback, birds were exposed to another hour of silence with the lights extinguished and then perfused immediately to ensure maximum quantity and quality of ZENK preservation. 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. Birds were perfused via the left ventricle using heparinized 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA). The brain of each individual black-capped chickadee was then extracted and placed in a solution of PFA for 24 hours, followed by a 30% sucrose PBS solution for 48 hours. The brains were then fast frozen and stored at -80℃ until sectioned.

Histology

After being sectioned sagittally from the midline, 48 40µm sections of each hemisphere were collected and stored in 0.1 M PBS. In order to visualize ZENK, sections were first washed twice in 0.1 M PBS for a minimum of five minutes, transferred to a 0.5% H_2O_2 solution, and incubated for 15 minutes. Incubation was followed by three 5 min washes in 0.1 M PBS. A second incubation in 10% normal goat serum for 20 hours at room temperature followed. Sections were then transferred into the primary antibody (egr-1, Abcam Monoclonal Egr-1 ab133695; Abcam Inc, Toronto, ON, Canada) for 24 hours at a concentration of 1: 5,000 in 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 3 more washes in PBS/T, sections were incubated in avidin-biotin horseradish peroxidase (ABC Vectastain Elite Kit; Vector Labs, Burlington, ON, Canada) for one hour, followed by three washes in 0.1M PBS. Sections were then processed with 3,3'-diaminobenzidine tetrachloride (Sigma FastDAB, D4418, Sigma-Aldrich, Santa Fe Springs, CA, USA) to visualize expression of ZENK, followed by three washes with 0.1M PBS to remove any excess visualizing agents.

Imaging

Eight sections were mounted on a slide and cover slipped. Three neuroanatomical regions (caudomedial mesopallium; CMM, caudomedial nidopallium dorsal; NCMd, and caudomedial nidopallium ventral; NCMv) were subsequently imaged using a Leica microscope (DM5500B; Wetzlar, Germany) to analyze ZENK expression. Eight images of each region were taken per hemisphere, for a total of 48 images per subject. Images were taken 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). Overlap in the ventral and dorsal regions of the NCM was carefully avoided. ImageJ version. 1.46v (Image Processing and Analysis in Java; publish) was then used to quantify immunopositive ZENK cells.

Results

We conducted a repeated measures ANOVA using SPSS (IBM SPSS Statistics for Windows, Version 26.0. 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 (Male *Chick-a*, Female *Chick-a*, and Pink noise) and sex as between subject factors. As expected from previous studies, there was a significant main effect of brain region ($F_{(2, 16)} = 7.363$, $p = 0.005$) and hemisphere ($F_{(1, 8)} = 11.157$, $p = 0.01$).

There was a significant main effects of playback condition ($F_{(2, 8)} = 8.259$, $p = 0.011$). We then conducted a Tukey HSD-corrected pairwise comparison on playback condition with an alpha level set at 0.05. We found that the Pink noise condition was significantly different from the Male *Chick-a* ($p = 0.009$) playback group, but not significantly different from the Female *Chick-a* ($p = 0.066$) playback group (Figure 5.1a). We also found that the Male *chick-a* and Female *chick-a* groups were not significantly different from each other ($p = 0.193$). We did not

find any significant effects of Sex of the receiver ($F_{(1, 8)} = 0.0$, $p = 0.991$) or any significant interactions (Figure 5.1b).

Discussion

We found that both male and female chickadees respond similarly, in terms of their ZENK protein response, to both male and female *chick-a* calls at a neurobiological level. This suggests that there is no bias towards calls of either sex, at least in the *chick-a* portion of the *chick-a-dee* call. While we only found a significant difference in protein expression between the Male *chick-a* group and the Pink noise control group, the Female *chick-a* group was trending towards significance when compared with the Pink noise group ($p = 0.066$). As the Pink noise group was comprised of only one bird of each sex, for a total n of 2, it is possible that the limited sample size restricted results.

Some of the first research done examining the function of the black-capped chickadee *chick-a-dee* call examined the bioacoustics (i.e., characterized the acoustic properties) of each note type. After first understanding the semantics of the call, Hailman, Ficken, and Ficken (1987) then expanded on the importance of the D notes, suggesting that D notes play a separate role in the call than a combination of the other note types. With D notes being acoustically different from the other note types, it was suggested that they may encode for separate information than the rest of the call (Hailman et al., 1987). Charrier, Bloomfield, and Sturdy (2004) conducted an extensive bioacoustic analysis of each note type measuring 10 features of non-D notes and found that 9 out of 10 of these acoustic features differed significantly between all note types. Descending duration was the only acoustic feature that was not different among note types and no sex differences were detected for any note type (Charrier et al., 2004). However, a recent and more extensive study revealed that in fact A notes do contain some sex specific information

(Campbell et al., 2016). Interestingly, sex specific information appears only in A notes, as this note type is also thought to be the only unlearned note in the *chick-a-dee* call (Hughes, Nowicki, & Lohr, 1998). Since our stimuli used randomly selected vocalizations, it is possible that not all of our stimuli contained equal numbers of each note type, making some stimuli easier for obtaining information on the sex of the caller than others.

Building from the bioacoustics, operant go/no-go experiments have also been used to examine how chickadees perceive the call note types and whole calls. An important first step by Sturdy and colleagues (2000) showed that black-capped chickadees do categorize the four *chick-a-dee* call note types as separate open-ended categories. Since then, many studies have used full *chick-a-dee* calls to show how the call contains information regarding species (Bloomfield et al., 2003) and threat level (Templeton et al., 2005). Arguably just as important, studies have also shown how chickadees do not seem to use the *chick-a-dee* call to distinguish between seasons (Scully et al., 2019) or sex (Campbell et al., 2020). Our findings here support the behavioural results Campbell et al. (2020) that suggests while birds are able to learn to categorize both full *chick-a-dee* calls and just the *chick-a* portion of calls by sex, they do not generalize this learning to untrained calls, suggesting that discrimination of caller sex is not a natural function of the *chick-a-dee* call.

At a neurobiological level, *chick-a-dee* calls are processed in the same auditory areas as all other vocalizations, the NCM (dorsal and ventral) and CMM. An important study on the effects of sex found that both male and female chickadees had more ZENK labeled cells in the CMM and NCMd in response to hearing male *chick-a-dee* calls than female calls (Avey et al., 2008). This suggests that birds are using an acoustic feature within the call to identify the sex of the caller. While previous studies have examined how these brain areas respond to full *chick-a-*

dee calls (e.g., Avey et al., 2011; Scully, 2018), few have examined individual aspects of the call. Avey and colleagues (2014), used just the D notes to investigate the effect of conspecific versus heterospecific simple calls on neural expression. By using only the D note, the call was more acoustically similar to the calls of heterospecifics and demonstrated that there was no difference in the amount of ZENK expression induced by conspecific or heterospecific calls (Avey et al., 2014). In combination with a previous behavioural study that found chickadees use the *chick-a-dee* call for species discrimination (Bloomfield et al., 2003), Avey and colleagues (2014) neurobiological study then suggested that some component in the *chick-a* portion of the call is responsible for driving this categorization.

Previous studies have focused on either the full *chick-a-dee* call or D notes, here we narrowed in on the beginning of the call. The stimuli used in the current study, as well as in Campbell et al.'s (2020) study were created by manually removing the *dee* portion of a full *chick-a-dee* call, Therefore, we are not able to generalize our findings to naturally produced *chick-a* calls. As chickadees have been shown to produce just *chick-a* calls, our altered stimuli may not contain the same information that these calls are used for in the wild. Thus, the next step to understanding the function of *chick-a* calls must then be to use naturally produced calls.

Conclusions

Overall, our results showed no neurobiological difference in the perception of male and female *chick-a* calls. Although A notes contain some information regarding the sex of the caller (Campbell et al., 2016), this information is not attended to in the context of a *chick-a* call. Mirroring the findings of a behavioural study, we found that male and female chickadees react similarly to both calls of the same and different sex as listeners. It is possible that while information regarding an individual's identity is located within the *chick-a-dee* call, the function

of the call does not require knowing an individual's sex. Future studies should focus more closely on the *chick-a* call, moving from when it is produced to why.

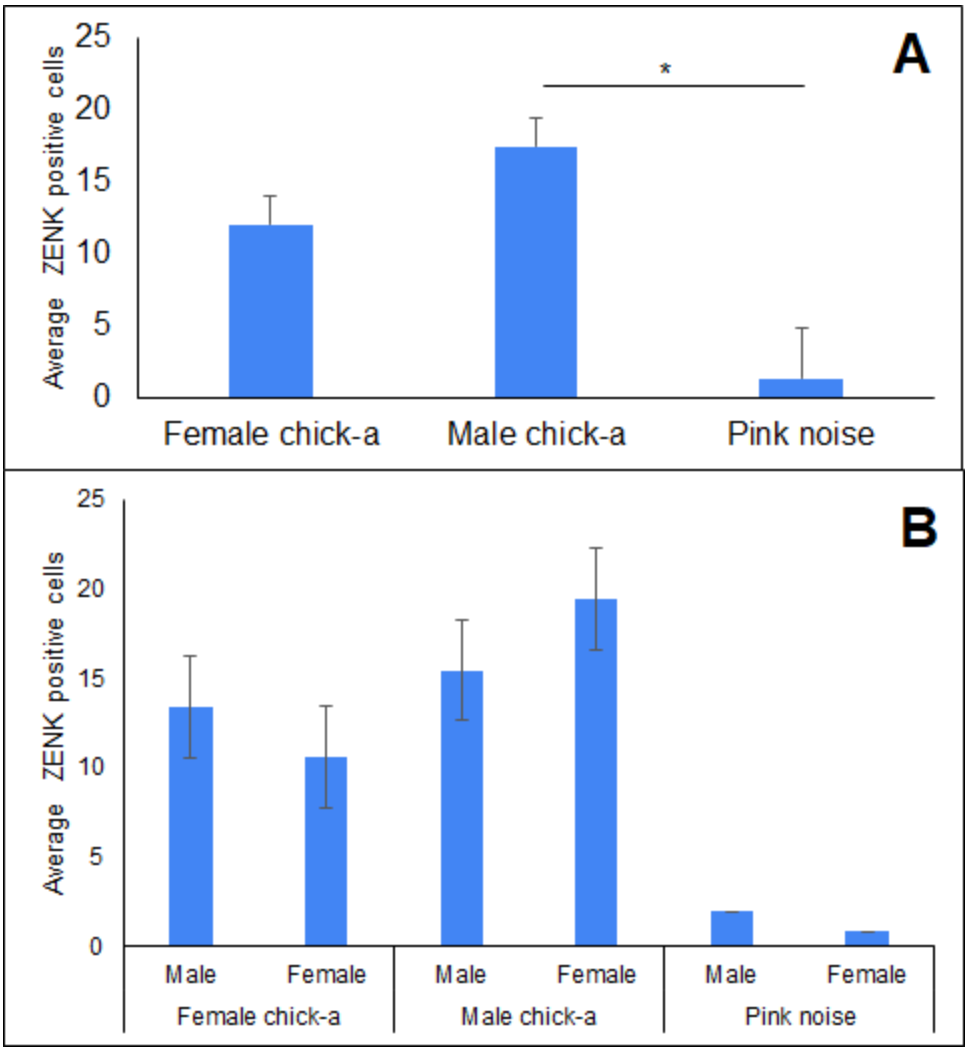

Figure 5.1: Neural expression per group. A) Average ZENK positive cells labeled per playback group. * indicates significant difference between the Male *chick-a* and Pink noise groups. B) Breakdown of playback group by sex of the listener.

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Chapter 6: General Discussion

Summary of data chapters

This thesis examined the perception of black-capped chickadee *chick-a-dee* calls from both a behavioural and neurobiological perspective. First, in Chapter 2, I conducted an operant go/no-go discrimination task to examine whether chickadees can categorize calls produced in different seasons. I found that while chickadees could learn to discriminate calls produced in either fall or spring, birds did not generalize (i.e., categorize) this learning to calls not used for training, suggesting that season of production is not a feature in the *chick-a-dee* call to which birds attend. In Chapter 3, I modified a previously conducted behavioral study into a neurobiological study to examine whether chickadees respond differentially to high or low duty cycle (or the amount of time taken up by vocalizations) *chick-a-dee* calls in the auditory nuclei used to perceive vocalizations. Although the previous behavioural study found that chickadees respond more vigorously to calls delivered with higher duty cycles, I found that there was no difference in the amount of ZENK labeling between calls delivered with high and low duty cycles. Next, in Chapter 4, I tested multiple Egr-1 primary antibodies as possible replacements in songbird research for the recently discontinued standard, Santa Cruz Egr-1 sc-189 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Using the same protocol as previously used in my laboratory, I identified one primary antibody, at two different concentrations, that had specific labeling of the Egr-1 (or ZENK) protein in the songbird auditory areas. In Chapter 5, I used the primary antibody trialed in Chapter 4 to identify whether chickadees attend to information regarding sex of the caller in the *chick-a* portion (A, B, and C notes) of the *chick-a-dee* call at a neurobiological level. Neither male nor female chickadees differed in ZENK labeling after hearing either male or female *chick-a* calls suggesting conveying sex of caller is not a main

function of these calls. Combined, I demonstrate how behavioural and neurobiological studies can be used in concert to further our understanding of the perception of vocalizations.

Neuronal activation via ZENK labeling

ZENK (an acronym for zif268, Egr-1, NGFI-A, krox24) is the protein product of the immediate early gene (IEG) *zenk*. IEGs are rapidly transcribed after cell depolarization, with or without *de novo* protein synthesis, using the cell's preexisting transcription factors (Watson & Clements, 1980). Like most IEG protein products, ZENK is a transcription regulatory protein involved in long-term cellular changes for memory and learning (Watson & Clements, 1980; Jarvis & Nottebohm, 1997). ZENK is used in multiple animal models as a way to visualize neural activity in response to sensory activation. In songbirds, ZENK is used most often to study perception and production of vocal communication (Knapska & Kaczmarek, 2004).

The caudomedial mesopallium (CMM) and the caudomedial nidopallium (NCM) are the two main areas studied among the auditory nuclei of the songbird brain. Though most auditory information is first processed in Field L of the telencephalon, a subsection of this area, L2a, does not express the *zenk* gene, leading researchers to instead focus on CMM and NCM, which both receive input from Field L and robustly express *zenk* (Mello et al., 1992; Park & Clayton, 2002; Ribeiro et al., 1998). The songbird community, and our laboratory group, has taken advantage of the ability to label ZENK in CMM and NCM to expand our knowledge of how conspecific and heterospecific calls are perceived in both zebra finches and black-capped chickadees (e.g., Scully et al., 2017; Avey et al., 2014). Researchers have also been able to further understand how perception can differ based on sex (Gobes et al., 2009), threat (Avey et al., 2011), and rearing histories (Hahn et al., 2015), to name a few salient examples.

However, during the completion of this dissertation, a major obstacle developed. The primary antibody, widely accepted as the standard used in the field, made by Santa Cruz Biotechnologies Egr-1 sc-189 (Santa Cruz, CA, USA), no longer specifically labeled ZENK positive cells (unpublished observations, birdsong-l@usc.edu [Electronic mailing list]). Following this, Santa Cruz discontinued production of the Egr-1 antibody all together, creating a demand for a new primary antibody in the field. In Chapter 4, I tested multiple antibodies to find a replacement that specifically labeled ZENK using our existing protocol. I tested two novel Egr-1 antibodies, at multiple concentrations, along with an antibody for the IEG cFos, which has similar expression patterns to ZENK (Mello, Velho, & Pinaud, 2004). The Egr-1 antibodies from Proteintech (Proteintech polyclonal Egr-1 55117-1-AP) and the cFos antibody (Abcam c-Fos ab209794) were found to have non-specific labeling; however, the Egr-1 antibody from Abcam (Abcam Egr-1 ab133695) was found to selectively label cells in the auditory areas in a similar fashion to previous studies, but only at lower concentrations. Since all the testing of antibodies was conducted using zebra finches the selected antibody, Abcam Egr-1 ab133695, was further tested, and verified to work, with black-capped chickadees.

In Chapter 5, I used the antibody found in Chapter 4 to label ZENK cells in response to playback of male and female *chick-a* calls. Using both male and female black-capped chickadee produced *chick-a* calls, I measured the number of cells positively labeled for ZENK in the CMM and NCM in both male and female chickadees. There was no statistical difference in the amount of ZENK between male and female listeners. There was significantly more ZENK in response to hearing male *chick-a* calls than in the control group that heard pink noise, which is consistent with previous studies using the old, now discontinued, antibody (Avey et al., 2005; Gobes et al., 2009). While the birds who heard female *chick-a* calls did not produce significantly more ZENK

than the control group, the difference approached significance ($p = 0.066$), and followed the trend of previous studies. Taken together, the results of Chapter 5 help to further validate the findings of Chapter 4, while also answering a research question of its own, namely, are there any sex differencs in ZENK expression to *chick-a* call playback in male and female black-capped chickadees.

In spite of the fact that Chapter 3 used the original ZENK antibody from Santa Cruz, the results of this experiment can still be used to identify how calls are perceived. In Chapter 3, I aimed to find out if chickadees respond differently to *chick-a-dee* calls produced at different duty cycles. A duty cycle refers to the amount of time taken up by a vocalization, for example a high duty cycle may have 10 calls within 10 seconds while a low duty cycle would have two calls within 10 seconds. If there was a difference in ZENK labeling, then that could suggest that perception of calls depends on not just information contained within the call, but the context (i.e., rhythm or beat) in which the call is presented. I found that there was no difference in the amount of ZENK labeled cells across duty cycles. Since I did not find a difference between high and low duty cycle calls, this suggests that perception at a neurobiological level, at least in the CMM and NCM, is more specialized to individual calls rather than the context.

In Chapters 3,4, and 5, I used a neurobiological approach to study how chickadees perceive *chick-a-dee* calls. Though Chapter 4 was a methodological study, Chapters 3 and 5 expanded directly our understanding of what signals and features of signals are and what signals are not processed in the CMM and NCM. Comparing the results of Chapter 3 and 5 offer yet another way of showing consistency between the old, Santa Cruz antibody and the new, recently validated Abcam antibody. Using both primary antibodies we found the most ZENK expression in the CMM followed by the dorsal portion of NCM (NCMd), with the least expression in the

ventral portion of NCM (NCMv). One difference that we did notice between the two antibodies was the total number of cells labeled. Though the trends of labeling were the same regardless of antibody used, we noticed that brains labeled with the new, Abcam antibody labeled approximately half of the total number of cells labeled in brains treated with the Santa Cruz antibody. This may be due to the novelty of this antibody, which may need more experimenting with the ideal concentration for maximal labeling. Another possible limitation with the use of the Abcam antibody is that we have only tested it on auditory areas, where many researchers use ZENK in other sensory areas of the songbird brain. In addition, I only tested the antibodies using the preexisting protocol used in our laboratory. Other researchers who use other protocols may find different results, thus we cannot definitively say that the other antibodies tested will never work.

Combining approaches

A common theme throughout my thesis has been combining multiple approaches to converge on the same question. Most of the experiments I conducted were neurobiological studies expanding on previous behavioural studies. I also conducted my own behavioural operant go/no-go study and supported by findings by conducting a bioacoustic analysis. I truly believe that viewing a question from multiple angles is necessary to fully understand how something works.

In Chapter 2, I conducted an operant go/no-go discrimination study to examine if the season of which a *chick-a-dee* call is produced can be categorized by chickadees. I found that while the chickadees were able to learn the categories of calls produced in fall versus calls produced in spring, they did not generalize their learning to untrained stimuli. These results led me to ask if there were any differences between these calls at a bioacoustic level. After

measuring four different acoustic features, I found that there were no major bioacoustic differences between calls produced in the fall versus calls produced in the spring. However, I did find that one measurement, the number of notes per call, was significantly different between seasons. This could suggest that if there are any differences within calls across seasons it could be at the individual note level, as we did not measure acoustic differences between note types. Overall Chapter 2 used bioacoustics to help understand behavioural results, and direct future research.

A previous study by Willson and Menill (2011), asked whether chickadees respond differently to calls differing in duty cycle. They found that chickadees would approach the speaker more in response to high duty cycles than to low duty cycles. Based on these findings, in Chapter 3 I ask if this difference in response was also present at a neurobiological level. Using the same stimuli played in the behavioural study, I measured the number of ZENK positive cells in the CMM and NCM after playback. Surprisingly, there was no difference in ZENK amounts between high and low duty cycles. Unlike in Chapter 2 where results from the two approaches agreed, here the two approaches gave us differing answers suggesting that perhaps this preference develops outside of the two auditory nuclei examined in Chapter 3.

In Chapter 5, I again replicated a behavioural experiment using a neurobiological lens. Previous bioacoustic studies contradicted each other regarding sex differences across note types in the *chick-a-dee* call (Campbell et al., 2016; Charrier et al., 2004). To address this confusion, Campbell and colleagues (2019) used an operant conditioning go/no-go discrimination task to examine if black-capped chickadees could tell the difference between both male and female full *chick-a-dee* calls and cut *chick-a* calls. The behavioural study found that while chickadees are able to learn to discriminate between male and female calls (both full and cut), they did not

generalize this learning to untrained calls. While the behavioural results supported one of the bioacoustic studies which found that there are no noticeable sex difference in *chick-a-dee* calls, I wanted to see if there were any differences at a neurobiological level. As the only part of the call that had been proposed to contain information regarding an individual's sex was the A note (Campbell et al., 2016), I focused on the *chick-a* portion of the *chick-a-dee* call. After playback of both male and female chick-a calls, there was no difference in the amount of ZENK labeled cells between sexes of listeners or callers. Unlike in Chapter 3, these neurobiological results are similar to the behavioural results. While our results support the idea that chickadees do not attend to a caller's sex when listening to *chick-a-dee* calls, our results also suggest that there is some difference between the sexes in the calls produced. Campbell and colleagues (2019) showed that chickadees were able to learn the categories of male and female, and in Chapter 5 I did find slight differences in ZENK labelling in response to sex. While not significantly different from each other, birds who heard male *chick-a* calls had more ZENK labeled cells than those who heard female *chick-a* calls. In addition, only the male *chick-a* group was significantly different from the control pink noise group, again suggesting potential preceived differences.

In three of my four chapters, I used novel approaches to answer questions to fill in knowledge gaps left by previous investigations. In Chapter 3 I used a neurobiological approach to answer questions left by a behavioural study. Surprisingly, I found that the neurobiological results differed from the behavioural results, leading to more questions for future research. In Chapters 3 and 5, the results of multiple approaches complimented each other, allowing for a broader understanding of how chickadees perceive *chick-a-dee* calls. The concept of integrating multiple approaches to answer the same question is not novel. Following the results of Templeton et al. (2005), who found that chickadees respond with more D notes to high threat

predators than low threat, Avey et al. (2011) measured ZENK in birds that heard calls produced in response to high versus low threat predators. The neurobiological study showed that chickadees produced more ZENK positive cells to calls produced to high threat than low threat predators (Avey et al., 2011), complementing the behavioural results. More recently, a hormonal study found that dopamine and serotonin release triggered by vocalizations changes across seasons (Rodriguez-Saltos et al., 2018). These findings provided an neurobiological explanation for results of another study that found that mobbing call rates changed depending on season, suggesting a neurobiological factor to the behavioural results (Dutour et al., 2019). Combining methodology and addressing a question from multiple approaches is an important step in science to better understand the world around us.

Conclusions

The goal of this thesis was to use neurobiological and behavioural approaches to expand the understanding of how black-capped chickadees perceive *chick-a-dee* calls. These studies showed how neural and behavioural responses to the same acoustic stimuli can differ depending on context. Chapter 2 showed that while use of *chick-a-dee* calls differ across seasons, birds do not categorize calls as different based on season of production, nor are there major bioacoustic differences in calls. Chapter 3 showed how behavioural responses to a stimulus may not be dictated by the auditory processing areas, at least regarding duty cycles. Chapter 4 differed from the rest of the thesis, focusing on replacing a previously gold-standard antibody with a new option for visualizing ZENK. Chapter 5 demonstrated the validity of the new antibody, while complementing previous behavioural and bioacoustic studies to show that sex of a caller is not attended to by chickadees. Future work should continue to use a multi-approach method to expand on how songbirds perceive vocalizations.

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