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The neural mechanisms underlying the perception and production of learned vocalizations in songbirds

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Abstract

Songbirds produce a wide array of vocalizations, including song, and learned and innate calls. Songs and calls can be functionally defined. Songs are typically used to attract potential mates and defend one's territory, whereas calls are used for everything else, such as advertising the presence of a predator, or location of a food source, and maintaining contact with members of one's flock. The purpose of this thesis was to better understand the neural mechanisms underlying call production and perception in two songbird species; the black-capped chickadee (*Poecile atricapillus*) and the zebra finch (*Taeniopygia guttata*). My objectives were to (1) understand the involvement of the song-control system in the production of calls (Chapter 2, 3), (2) understand how bird calls are perceived in the brain (Chapter 4), (3) and if the song-control system is involved in the neural basis of perception of bird calls (Chapter 5). Black-capped chickadees were used to examine the motor-driven immediate-early gene (IEG) expression in the song-control nuclei, HVC and the robust nucleus of the arcopallium (RA). Chickadees that produced primarily *gargle* calls, an aggressive vocalization used in antagonistic encounters had the most IEG expression in HVC and RA, therefore are involved in the production of calls in chickadees. Chickadees were subjected to HVC lesions, and their *gargle* and *chick-a-dee* calls were compared pre- to post-lesion. The *gargle* calls were shorter, much more variable and were missing several notes post-lesion, whereas the *chick-a-dee* calls were also affected but not to the same degree. Therefore HVC is crucial for the normal production of the *gargle* and *chick-a-dee* calls. To explain this neural basis of perception of learned calls, chickadees were exposed to *fee-bee*, *gargle*, *chick-a-dee* and *tseet* vocalizations and IEG expression was examined in the auditory forebrain. The *gargle* elicited the most IEG expression. Finally intact male and female zebra

finches, as well as HVC lesioned males were exposed to female and male long-calls and IEG expression in the auditory forebrain was measured. The auditory forebrain showed more IEG expression for male long-calls only in HVC lesioned males. Overall these results indicated the integral function of the song-control system in call production and perception, and would suggest that these structures should be collectively called the vocal-control system.

Keywords

HVC, black-capped chickadee, learned call production, zebra finch, lesion, ZENK immunoreactivity, *gargle*, *chick-a-dee*, long-call

Co-Authorship Statement

Portions of Chapter 1 were published: Mischler, SK, Congdon, JV, Scully, EN, Campbell, KA, & Sturdy, CB. (2017). Passerine vocal communication. *Encyclopedia of Animal Cognition and Behaviour*, 1-7. Jenna Congdon, Erin Scully and Kimberley Campbell all wrote sections of this encyclopedia entry, and Christopher Sturdy and I edited the entire manuscript. Portions of Chapter 1 were used in sections where I was the primary author.

Chapter 2 will be submitted for publication. Scott MacDougall-Shackleton will be a co-author. He contributed to the experimental design, the statistical analyses, edited the manuscript, and provided funding for the project. I designed the experiment, conducted all experimental procedures, analyzed data, and wrote the manuscript.

Chapter 3 will be submitted for publication. Scott MacDougall-Shackleton and Opal Sekler will be a co-authors. Scott contributed to the experimental design, the statistical analyses, edited the manuscript, and provided funding for the project. Opal assisted with HVC lesion surgeries, and immunohistochemistry, as well as providing rough notes for much of the background research. I designed the experiment, conducted all experimental procedures, analyzed data, and wrote the manuscript.

Chapter 4 will be submitted for publication. Scott MacDougall-Shackleton will be a co-author. He contributed to the experimental design, the statistical analyses, edited the manuscript, and provided funding for the project. I designed the experiment, conducted all experimental procedures, analyzed data, and wrote the manuscript.

Chapter 5 will be submitted for publication. Scott MacDougall-Shackleton will be a co-author. He contributed to the experimental design, the statistical analyses, edited the manuscript, and provided funding for the project. I designed the experiment, conducted all experimental procedures, analyzed data, and wrote the manuscript.

Chapter 6 was written by myself and is not published.

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"*Intelligence without ambition is a bird without wings*".

Salvador Dali

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List of Abbreviations

Av: nucleus avalanche

CMM: caudomedial mesopallium

HVC: used as proper name, formerly the high vocal center

f_0: first visible harmonic

F_{max}: loudest frequency

PBS: phosphate buffered saline

PBST: triton in phosphate buffered saline

PC: percent change

$$\frac{\{\text{Mean value of measure pre-lesion} - \text{Mean value of measure postlesion}\}}{\text{Mean value of measure pre-lesion}} \times 100$$

RA: robust nucleus of the arcopallium

NCMd: dorsal caudomedial nidopallium

NCMv: ventral caudomedial nidopallium

NPF: note peak frequency

nXIIts: tracheosyringeal portion of the hypoglossal nucleus

ZENK: zif268, egr-1, NGFI-A, Krox24

Chapter 1

1 Introduction and Literature Review

One of the most commonly studied phenomena in animal behaviour is that of vocal learning and vocal production in oscine birds, focusing specifically on birdsong. This is partly because it is a trait that is not only conspicuous, but often times elaborate and variable in its production. In addition, birdsong and human speech share a variety of similarities, both in the timeline of the development and acquisition of vocalizations and in the neural mechanisms underlying this process. In contrast to birdsong, the neural mechanisms underlying bird calls have for the most part been ignored, as it was believed they were innate therefore not subject to modulation by the song-control system (the network of nuclei responsible for the learning and production of song). However bird calls are an ideal candidate for study since many of them are learned and they are crucial for the animals' survival. In this chapter I will review the fundamental differences between birdsongs and calls, and the neural mechanisms that are involved in the learning, production and perception of birdsong, and how the neural mechanisms of call production and perception have largely been ignored. I used black-capped chickadees (*Poecile atricapillus*) and zebra finches (*Taeniopygia guttata*) for my studies and will discuss why they are ideal study species to investigate the neural mechanisms of bird calls. In this thesis my main objective was to try and understand the involvement of the song-control system in the production and perception of bird calls in black-capped chickadees and zebra finches.

1.1 Songbirds as model systems

Songbirds have increasingly been used in order to study the mechanisms that underlie vocal communication and imitative vocal learning, and as a model of human speech development (Doupe & Kuhl, 1999; Slater, 2003). In psychological research, the primary animal model that is used is the rat. However, songbirds allow us to investigate different questions. Songbirds are unusual in that they possess a vocal organ that allows them to produce elaborate vocalizations; as a group, they are also comprised of a large number of related species that vary in their vocal learning abilities. This variation among species allows comparative analyses on species that are suited to psychological studies. Songbirds are typically small, easy to house in captivity, have high metabolisms, and are able to be used to compare the underlying neural mechanisms across species (see Kroodsma & Miller, 1996). Songbirds are an ideal species to use for comparative studies; they learn and produce their vocalizations in a similar way to how humans learn speech.

The most widely studied species of songbird, especially in terms of neural mechanisms of singing behaviour and perception, is the zebra finch. The zebra finch is native to forests and grasslands in Australia; they are sexually dimorphic, with males and females showing different patterns in their plumage and colouring (Zann, 1996). Male zebra finches also learn their complex song from tutors (typically the father), whereas females do not sing. Zebra finches also produce a variety of other vocalizations, which are described in greater detail below. Males and females both produce a contact call, however this call is learned in males, and innately produced in females (Simpson & Vicario, 1990). Treating female finches early in life with male hormones leads them to produce more male-typical calls (Simpson & Vicario, 1991). Although female calls are

innate, we know that they are in fact individually different, and can be used to identify a particular individual (Forstmeier, Burger, Temnow, & Deregnaucourt, 2009). The fact that only males learn their vocalizations does limit the extent to which we can use zebra finches as a model system, but the vast amount of research conducted provides us with a large knowledge base to investigate different aspects of vocal communication.

Black-capped chickadees are also a useful songbird model for vocal learning, especially when studying in North America. Chickadees are widely distributed across most of Canada, as well as parts of the northern United States of America, stretching from the east to the west coast (Smith, 1991). Unlike many North American birds, they do not migrate; they eat seeds and insects, and are sexually monomorphic. In the spring, black-capped chickadees form relatively monogamous pairs during the breeding season, and males will aggressively defend their territories. During the winter months, the birds tend to form flocks with a highly structured social dominance hierarchy (Smith, 1991). Black-capped chickadees are a useful model species because they are readily available, they are small enough to maintain in a laboratory environment, their vocalizations have been thoroughly documented, as well as their natural history, and unlike zebra finches, both males and females sing and produce learned calls (Hahn, Krysler, & Sturdy, 2013). Black-capped chickadees produce a variety of vocalizations including the *gargle*, *chick-a-dee* and *tseet* calls as well as *fee-bee* song (see Ficken, Ficken, & Witkin, 1978 for complete repertoire). And like many songbirds, young chickadees must learn their vocalizations from adult conspecifics (Ficken, Ficken, & Apel, 1985; Guillette, Bloomfield, Batty, Dawson, & Sturdy, 2011; Hughes, Nowicki, & Lohr, 1998; Shackleton, & Ratcliffe, 1993). Because of these various vocalizations, we are able to

study the underlying neural mechanisms in vocal production in the chickadee. Chickadees produce a wide array of acoustically complex vocalizations in addition to their song, and these calls also show evidence of learning (discussed in section 1. 3 Bird Calls). One important aspect to note about chickadee song, is that unlike most songbirds which produce structurally complex songs and simple calls, chickadees produce a relatively simple *fee-bee* song, comprising two notes (see Figure 1-2, lower panel) (Ficken et al., 1978). Other closely related Parids like the willow tit, *Poecile montana*, or the marsh tit, *Poecile palustris*, also produce simple songs consisting of one or two different note types, which can be repeated (Broughton, 2009). And like many other Parid species, the black-capped chickadee's calls are more complex, and most vocalizations are produced by both sexes, making them an ideal candidate in which to study the subtleties of the neural mechanism underlying these vocalizations.

1.2 Birdsong

Song is often an elaborate and complex vocalization and has three potential purposes. These are: to advertise and defend one's territory; to attract potential females for mating; and also potentially stimulating female reproductive behaviour and physiology (Catchpole & Slater, 2008; Kroodsma & Miller, 1996). Birdsong can also be stereotyped, in this case, it encompasses the notes, syllables and phrases, and also dictates the way in which song and song repertoires are delivered (Marler, 2004; Vicario, 2004). This type of song presentation can be quite formal; there is rhythmicity to singing and the progression through a song repertoire.

Songbirds are one of the few taxa to engage in vocal learning, similarly to how humans, cetaceans, bats, elephants, parrots and hummingbirds learn their vocalizations

(Doupe & Kuhl, 1999). In order for birdsong to be acquired there has to be a predisposition to learning as well as the experience of being exposed to song in order for vocal development (Brainard & Doupe, 2002; Marler & Tamura, 1964). Birdsong must be learned, and this process is generally divided into two phases, the sensory phase and the sensorimotor phase, which can overlap (see Figure 1-1) (Brainard & Doupe, 2002;

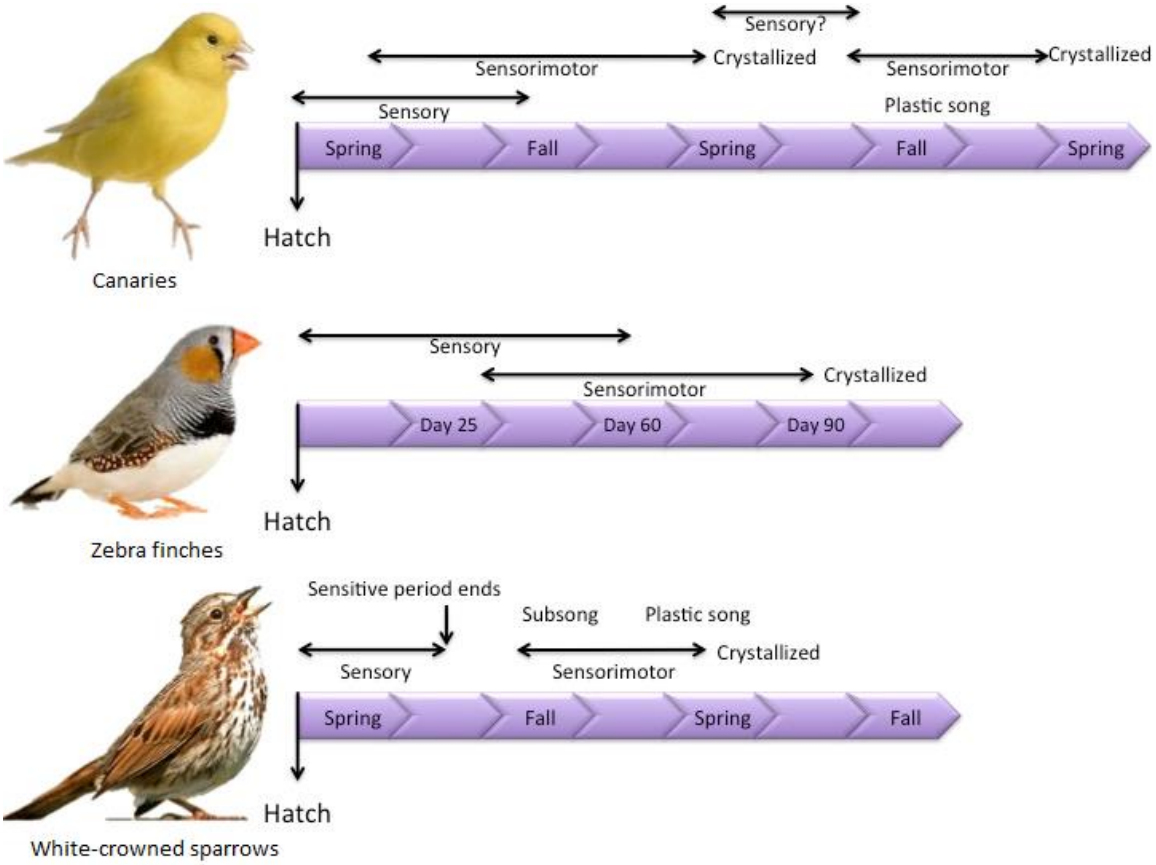

Figure 1- 1 Figure depicting the different types of song-learning and their respective timelines. Image adapted from Brainard & Doupe, 2002.

Kroodsma & Miller, 1996). During the sensory period, the songbird is in a sensitive period where the brain is prepared to receive auditory input. The songbird listens to the songs produced by adult songbirds (i.e., tutor birds), and their brain processes this auditory input and forms a memory template of song (Marler, 1997; Mooney, 1999). This input leads to both neural and behavioural changes, which leads into the sensorimotor

phase. In this phase, songbirds start to produce their own song based on the template that they formed or activated during the sensory phase. Initially this song is fairly inaccurate and variable, and is often compared to babbling in human infants (Aronov, Andalman, & Fee, 2008; Doupe & Kuhl, 1999; Prather, Okanoya, & Bolhuis, 2017). The auditory feedback that the songbird receives allows them to assess their performance and make changes to their song performances, until the song they produce matches the song template they developed during the sensory phase (Fee & Goldberg, 2011; Konishi, 1965). Songbirds can also generally be separated into two broad groups; open-ended learners and closed- ended learners (Brainard & Doupe, 2002; Catchpole & Slater, 2008; Slater, 2003). These two forms of song learning are described further below.

Early life experiences are crucial for song learning, and this learning can be disrupted in a variety of ways. The length of exposure to a tutor bird can severely impact birdsong (i.e., shorter exposures lead to less complex song structures) (Baptista & Morton, 1981; Thorpe, 1958). Acoustically isolating a bird from others during the sensory phase can lead to songs that are simpler, shifted in their frequencies and extremely variable (Marler, 1981; Marler & Peters, 1977; Shackleton & Ratcliffe, 1993). Preventing auditory feedback during the sensorimotor phase by deafening birds can also negatively impact song, resulting in shorter songs, delaying singing behaviour, or even eliminating song altogether (Konishi, 1965; Nottebohm, 1968). However, many species still maintain some of the features of their species-typical songs even when raised in isolation, indicating that there is partial encoding of some song features, or an inherent song template that initially directs song learning (Bolhuis & Gahr, 2006; Bolhuis,

Okanoya, & Scharff, 2010; Fehér et al., 2009; Marler, 1997; Searcy, Marler, & Peters, 1985).

In the thousands of species of songbirds on the planet, there is a huge amount of variation in the timeline of song learning (Beecher & Brenowitz, 2005). Despite this, the majority of research on song learning is conducted on the zebra finch (*Taeniopygia guttata*), the white rat of the bird world (Böhner, 1983; Böhner, 1990; Clayton, 1987; Clayton, 1988; Eales, 1985; Eales, 1987). For zebra finches, the sensory and the sensorimotor phases overlap (see Figure 1-1), these birds only produce one song type, and their song is crystallized (i.e., no longer changes) by 90 days of age, and does not change throughout adulthood (Slater, Eales, & Clayton, 1988). They are therefore considered closed-ended learners. However this form of song learning is only one end of the spectrum. Canaries (*Serinus canaria*), learn their song during the spring and practice it into the fall, and sing a crystallized song during the following spring (Nottebohm, Nottebohm, & Crane, 1986). They repeat this process every year; therefore their song repertoires expand and change annually. Therefore they are considered open-ended learners. Another developmental path is that of the white-crowned sparrows (*Zonotrichia leucophrys*), which learn their song in the first few months of life, but do not actually sing until the following breeding season (Marler, 1970). The two species of interest for this thesis are the zebra finch and the black-capped chickadee. The zebra finch and the black-capped chickadee are both closed-ended learners, which are characterized by the bird requiring sensory input early in life to produce a normal sounding song, however some aspects of this song (see Figure 1-2) (i.e., frequency) can be modulated in adulthood (Christie, Mennill, & Ratcliffe, 2004; Grava, Grava, & Otter, 2012; Hahn et al., 2013;

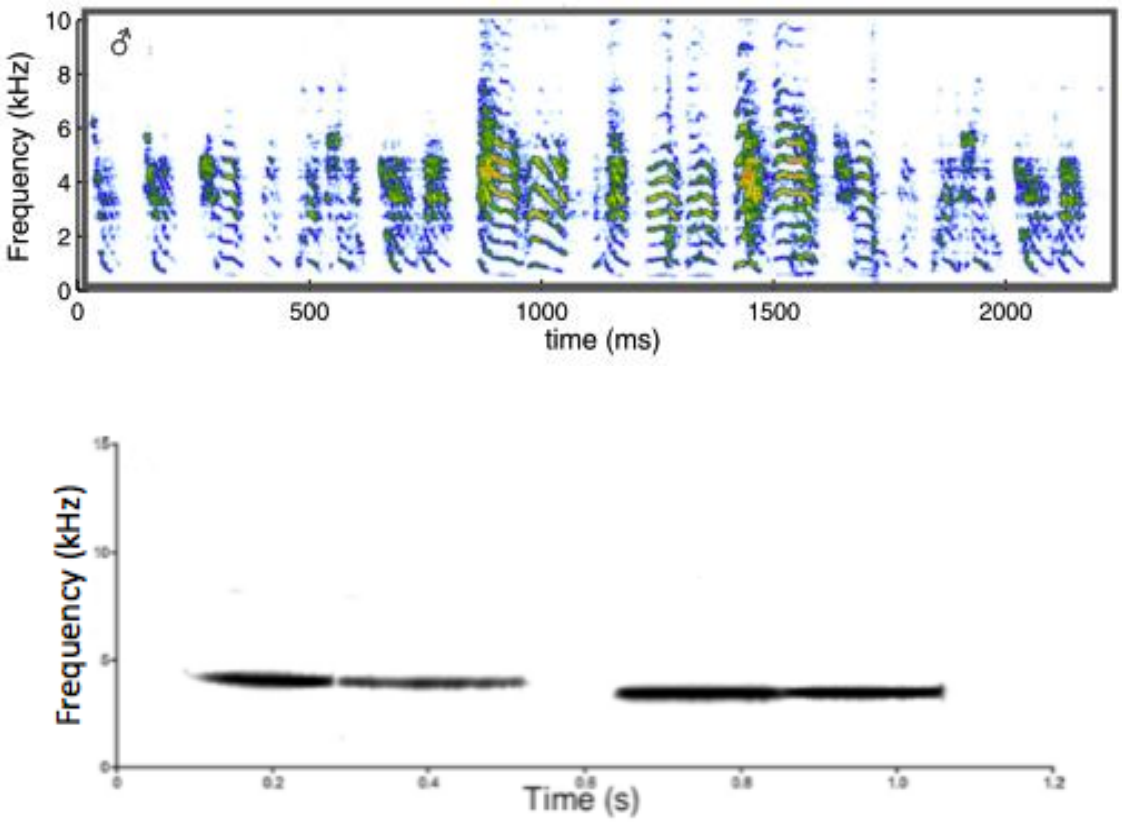

Figure 1- 2 Spectrograms of species typical zebra finch song and black-capped chickadee *fee-bee* songs. For both spectrograms the x-axis represents time, and the y-axis represents frequency. The top panel depicts the zebra finch typical song, spectrogram adapted from Elie & Theunissen, 2016. The bottom panel depicts the *fee-bee* song of the black-capped chickadee, adapted from Avey, Rodriguez, & Sturdy, 2011.

Ratcliffe & Weisman, 1985; Shackleton & Ratcliffe, 1993).

1.3 Bird Calls

Bird calls are often distinguished from song by a variety of characteristics, although in some species this distinction may be somewhat blurred. On a functional level, song is often defined as having a role in courtship and reproduction, and calls are defined as vocalizations serving other functions (Spector, 1994). However, other definitions distinguish songs from calls based on acoustic or other features. Songs, as mentioned

above, are usually multi-part sounds, and produced primarily by males during the breeding season (Marler, 2004; Smith, 1991; Vicario, 2004). Songs are used for the purposes of reproduction and territoriality, typically have an underlying stereotypy, and are produced in most species primarily by males. Calls are typically simpler, even monosyllabic, and are produced by both sexes, at all age groups, are used daily for the purposes of communication, and many calls are produced by both males and females. Calls have a variety of functions, crucial for bird's survival (Marler, 2004).

Most species of birds must maintain their social groupings, whether it is in the context of a mated pair, a flock, or a family. Most birds have some form of *contact call*, which allows them to remain in contact with one another during foraging. *Separation calls* are sometimes a variation of a contact call, or could be completely different, and are given when a bird loses contact with their group. As finding food is also crucial for a bird's survival, some birds also emit *food calls* which announce the presence of a food source and indicate to other birds in the group to come and feed. A subset of these calls are *begging calls*, which are mostly produced by chicks after hatching, and which induce the parents to feed their offspring. These calls often allow for nest/kin recognition by the parents, or for nest mates to recognize one another (Beecher, 1982; Beecher, Beecher, & Hahn, 1981; Leonard, Horn, Brown, & Fernandez, 1997; Ligout, Dentressangle, Mathevon, & Vignal, 2016; Medvin & Beecher, 1986; Rowley, 1980).

Aggressive calls are used in agonistic interactions between individuals; the calls often lead to conflict resolution between the individuals. *Alarm calls* are used to announce the presence of a predator or danger in the environment. There are a variety of alarm calls, which include *distress* calls and *mobbing* calls. Distress calls are typically

produced when the individual is in the grip of a predator (Charrier, Bloomfield, & Sturdy, 2004; Stefanski & Falls, 1972; Zachau & Freeberg, 2012). Conversely mobbing calls are used when a predator is detected nearby, and to attract other members of the group to harass or "mob" the predator in order to have them hunt elsewhere. There are also variations of mobbing calls that tend to code for the type of predator, or the threat level to the individual (Avey, Hoeschele, Moscicki, Bloomfield, & Sturdy, 2011; Carlson, Healy, & Templeton, 2017; Ellis, 2008; Griesser, 2009; Krams & Krama, 2002; Rae, Whitaker, & Warkentin, 2015; Suzuki & Ueda, 2013).

It is important to note that the functional terms for calls described above are general terms. In some cases the same vocalization may serve more than one function, depending on how it is produced or the context. For example, the *chick-a-dee* call (see below) can serve a variety of functions including being a contact call and an alarm call.

For a long time calls were believed to be innate, however this is not always the case; many calls are learned or partially learned (for review see Marler & Slabbekoorn, 2004; Vicario, Raksin, Naqvi, Thande, & Simpson, 2002). This learning is done through a process of vocal imitation, similarly to how birds learn song (Vicario, 2004). Unlike song, which is produced primarily during the breeding season, many calls are produced year round and are more easily elicited in laboratory conditions. Also many calls are produced by both sexes, unlike song, which is primarily produced by males in many species. Thus, studying calls allows us to look at the learning and development of vocalizations in females as well as males. Since I am investigating calling behaviour in black-capped chickadees and zebra finches, I review evidence for learning in some of their calls below.

1.3.1 Learned black-capped chickadee calls

1.3.1.1 *Gargle* call

The *gargle* call is one of the most acoustically complex vocalizations that the black-capped chickadee produces, and is more acoustically complex than its *chick-a-dee* call (Ficken & Popp, 1992) (see Figure 1-3). This call is produced during agonistic

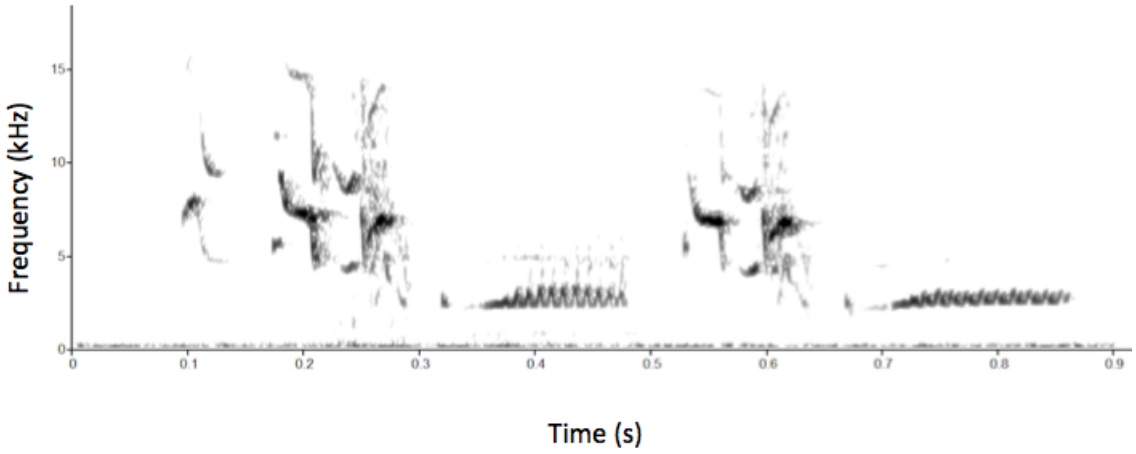

Figure 1- 3 Spectrogram of an example *gargle* call of the black-capped chickadee. The x-axis represents time and the y-axis represents frequency. Spectrogram obtained from personal recordings.

encounters between two chickadees and typically the caller is the winner of this interaction (Ficken, Weise, & Reinartz, 1987). These calls are also given year round, however recently they have been shown to have peak production during the summer months (Avey et al., 2011; Ficken, Ficken, & Witkin, 1978).

Similarly to most calls, the *gargle* was believed to be innate, and chickadees raised in acoustic isolation developed "normal" sounding *chick-a-dee* and *gargle* calls (Shackleton, & Ratcliffe, 1993). However this is no longer believed to be the case, chickadees found in different geographic regions produce different types of *gargle* calls,

and each individual chickadee has a repertoire of up to 10 different *gargles*, comprised of up to 10 syllables, therefore producing on average approximately 60 distinct *gargle* syllables (Baker, Baker, & Gammon, 2003; Baker & Gammon, 2008; Baker, Howard, & Sweet, 2000; Ficken, Ficken, & Apel, 1985; Ficken & Weise, 1984; Ficken et al., 1987). In a study by Baker and colleagues (2000) birds were sampled at three different locations (within 9 km of one another), and their *gargle* calls were compared across these different geographic regions. The *gargle* calls coming from the same location were far more acoustically similar than *gargle* calls produced from a different region. The component syllables were also more similar within the same population than between the different geographic regions. This would indicate that some form of learning occurs in the *gargle* call that allows the calls to differ significantly across small geographic regions. The component syllables of these calls are also very consistent across years, but the whole call itself is not as consistent, again suggesting that the call structures are affected by social and environmental interactions and learning.

The *gargle* call develops much later (after 40 days post-hatch) than the *fee-bee* song that develops in a high quality form, without any real intermediate phase between, days 20-30 post-hatch (Baker et al., 2003). It also develops later than the *chick-a-dee* call which follows a steady learning progression over the first 40 days of life. *Gargle* calls do not tend to match local *gargle* calls early on in life, but matched the *gargle* calls of where birds eventually settle, indicating that these calls may remain plastic for much longer, requiring vocal interactions with and imitation of local birds later in life (Baker et al., 2003).

1.3.1.2 *Chick-a-dee* call

The *chick-a-dee* call is another acoustically complex call, and is used for the purposes of expressing alarm when a predator is nearby, alerting other members of the flock to the presence of food, and coordinating flock movements (Ficken et al., 1978). This call is typically composed of four notes termed A, B, C and D, that are almost always given in this particular order (see Figure 1-4). The A, B, and C notes are rapid-

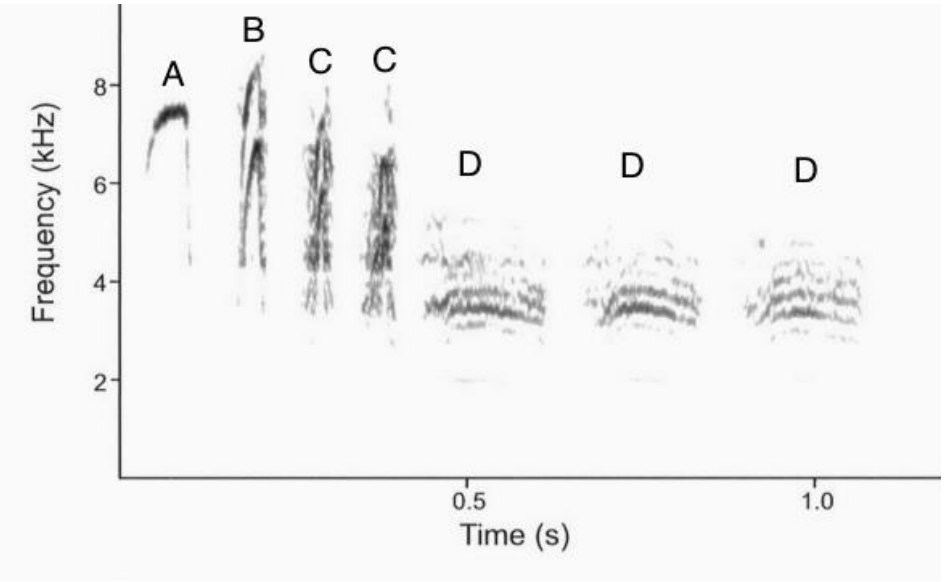

Figure 1- 4 Spectrogram of the black-capped chickadee *chick-a-dee* call. This figure depicts the four different note types which comprise the *chick-a-dee* call; A, B, C and D notes. Figure adapted from Charrier et al., 2004b.

frequency sweeps that form a structurally graded series (Ficken et al., 1978; Hughes, Nowicki, & Lohr, 1998). However within a particular call, each note can be repeated multiple times, just once, or omitted altogether (Ficken et al., 1978). The variable nature of the note repetition and combinatorial possibilities, allows for the coding of a huge amount of information within this call (Hailman & Ficken, 1986). *Chick-a-dee* calls can code for information about species identity (Bloomfield & Sturdy, 2008; Bloomfield,

Sturdy, Phillmore, & Weisman, 2003), individual identity (Charrier, Bloomfield, & Sturdy, 2004), and predator threat level (Templeton, Greene, & Davis, 2005).

There is some evidence suggesting that the *chick-a-dee* call is at the very least partially learned (Baker et al., 2003; Clemmons & Howitz, 1990; Hughes et al., 1998). Raising black-capped chickadees in social and acoustic isolation has a detrimental effect on *chick-a-dee* calls (Hughes et al., 1998). Birds raised in this social and acoustic isolation produce many fewer B and C notes, and, when they do produce these notes, they are acoustically different from normal B and C notes. Birds raised in social isolation, where they are housed in an individual cage but able to see and vocalize with birds their own age, show these same effects (Hughes et al., 1998). However when birds are raised with the social presence of an adult, or the presence of the parent birds, their *chick-a-dee* calls develop within the normal range. This indicates the crucial role of adult auditory input has on the development of at least some note types of the *chick-a-dee* call, which may be important for developing the sex specific characteristics of this call, particularly for the A note (Campbell, Hahn, Congdon, & Sturdy, 2016).

The components (A, B, C and D notes) of the *chick-a-dee* call do not all develop at once. Early in development chickadees produce a begging call, a signal to their parent to feed them. This begging call then develops and changes, and eventually becomes a D note when the chickadee reaches adulthood (Baker et al., 2003). It may be possible that the A, B and C notes develop later because they require more adult auditory input in order to develop normally.

A related species, the Carolina chickadee (*Poecile carolinensis*), shows geographic variation of the *chick-a-dee* call (Freeberg, 2012). Carolina chickadees from Tennessee and Indiana showed differences in their note compositions. Tennessee chickadees commonly produced D-hybrid (when an A, B or C note melds with a D-note) notes in their *chick-a-dee* calls, whereas this was a rare occurrence in Indiana chickadees (Freeberg, 2012). The *chick-a-dee* call serves a different purpose in each geographic location; for example Tennessee chickadees are less likely to use A notes in their *chick-a-dee* calls during flight, whereas this is not the case of Indiana chickadees. Similarly, Tennessee chickadees are less likely to produce D notes the closer they are to the group, and this is not the case for Indiana chickadees. The black-capped chickadee, being such a close relative of the Carolina chickadee, may likely show similar geographic variation in the use of notes, and the context in which this call is used, both of which seem to be learned from the local population.

Most of the evidence suggests that the production of the *chick-a-dee* call is at least partially learned; however the memorization, categorization, and discrimination of *chick-a-dee* calls may not be (Bloomfield, Farrell, & Sturdy, 2008). Black-capped chickadees captured as juveniles and raised with either conspecifics (black-capped chickadees) or heterospecifics (mountain chickadees), are able to discriminate between mountain and black-capped *chick-a-dee* calls. This suggests that black-capped chickadees possess an internal template for discrimination of *chick-a-dee* calls, which does not require input from adults within their own species (Bloomfield et al., 2008). Therefore, whereas memorization and auditory discrimination of the *chick-a-dee* call is not learned, production seems to be at least partially learned.

1.3.1.3 *Tseet* call

The *tseet* call of the black-capped chickadee is fairly simple acoustically, being composed of only one note at low amplitude, and is used for communication between chickadees at short distances (Ficken et al., 1978)(see Figure 1-5). The function of the

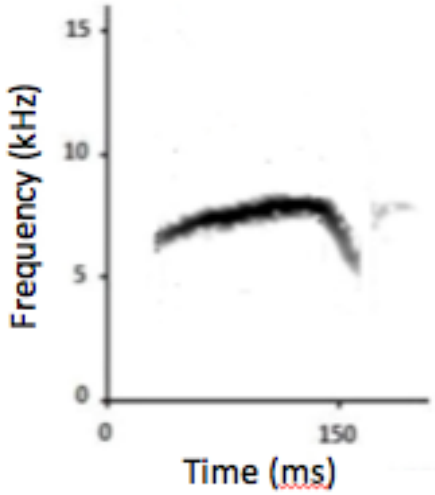

Figure 1- 5 Spectrogram of the *tseet* call of the black-capped chickadee, with time on the x-axis and frequency on the y-axis. Figure adapted from Guillette, Bloomfield, Batty, Dawson, & Sturdy, 2011.

tseet is not well understood, however it has been suggested that it is likely used to maintain pair or group integrity while foraging (Smith, 1991). The *tseet* call is also acoustically similar to the A note of the *chick-a-dee* call (Guillette et al., 2011). This call was initially believed to be innate, however it seems as though it may be partially learned (Guillette et al., 2011). Black-capped chickadees raised with mountain chickadees, or with no adult chickadees, showed differences in the starting frequency and descending frequency modulation of the *tseet* call compared to individuals raised with adult black-capped chickadees (Guillette et al., 2011). Therefore, acoustically simple calls are learned, and not innate as previously believed.

Although there is evidence for learning in the *gargle*, *chick-a-dee* and *tseet* calls, due to the ambiguous nature of the function and the acoustic simplicity of the *tseet* call, most of the projects in this thesis focus primarily on the *gargle* and *chick-a-dee* calls.

1.3.2 Learned zebra finch calls

Zebra finches are one of the most widely studied bird species in avian neurobiology, because they learn and memorize their song from a tutor bird, and this learning and memory process is similar to how human infants acquire speech (Funabiki & Konishi, 2003; Konishi, 1985). However in addition to song, they produce a variety of calls that are used in social contexts (Beckers & Gahr, 2010; Ter Maat, Trost, Sagunsky, Seltmann, & Gahr, 2014; Zann, 1996). The most commonly used calls in the zebra finch repertoire are the *tet*, the *stack* and the distance calls; also named the long-call or the contact call (see Figure 1-6), however this nomenclature has been inconsistent throughout the literature (Elie & Theunissen, 2016; Gobes & Bolhuis, 2007; Gobes et al., 2009;

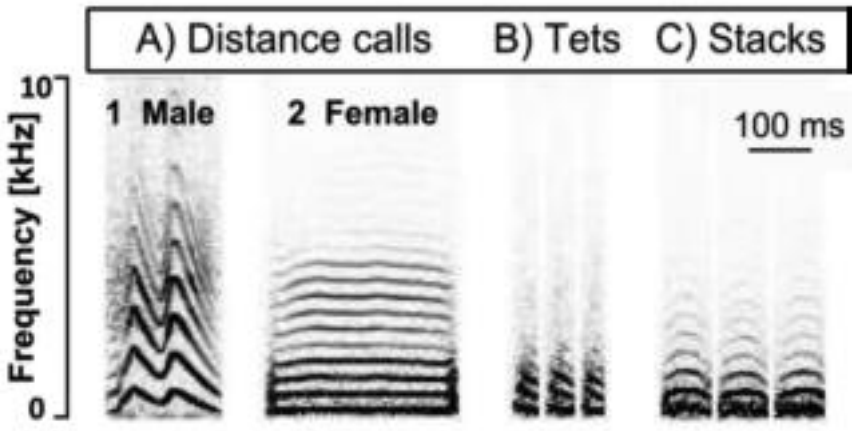

Figure 1- 6 Spectrogram depicting the three main types of zebra finch calls; the distance call, the *tet,* and *stack* calls. Legend is displayed on figure, representing time on the x-axis and frequency on the y-axis. Figure adapted from Gill, Goymann, Maat, & Gahr, 2015.

Vignal, Mathevon, & Mottin, 2004; Zann, 1984, 1985, 1996). *Tet* calls are probably the ones most used by zebra finches, and they may be involved in coordinating take-offs with family members during flight (Elie & Theunissen, 2016; Zann, 1996). *Tet* calls are primarily used as a short-distance contact call (Elie & Theunissen, 2016). *Stack* calls on the other hand tend to be longer and higher pitched than *tet* calls (see Figure 1-6), and are produced at the moment of take-off into flight, as well as during hovering bouts during flight (Zann, 1996). However, in this thesis I will be focusing on the distance call, and the evidence that this call is learned in males and not in females (Gobes et al., 2009; Marler, 2004).

1.3.2.1 Distance call in zebra finches

Distance calls (also called long-calls or contact calls) communicate a variety of information, including the caller's species, subspecies, geographic origin, sexual and individual identity (Okanoya & Dooling, 1991; Vicario, Naqvi, & Raksin, 2001; Zann, 1984). Distance calls are the loudest call given by the zebra finch, and can be heard from 80-100 m away (Zann, 1996; see Figure 1-6). It is given primarily when birds are isolated or scattered from one another, but is given in a wide variety of contexts as well: during mild alarm, stages of courtship, between singing bouts, as a greeting to newcomers, etc. Zebra finches typically form long-term relationships with their mates, and the distance call is often given when mates are separated from one another (Zann, 1996).

Distance calls are also sexually dimorphic; male and female distance calls are acoustically different; and males learn their distance call, whereas females do not (Simpson & Vicario, 1990; Vicario et al., 2001; Vignal, Mathevon, & Mottin, 2008; Zann, 1984, 1996). The female distance call is composed of a harmonic note, that

typically has a fundamental frequency around 500 Hz, the frequency is unmodulated, and the duration can vary but is typically longer than the male distance call (Simpson & Vicario, 1990; Vicario et al., 2001; Zann, 1984)(see Figure 1-6).

The male distance call also typically has a harmonic structure, and contains at least one of the following acoustic features: 1) a short duration, 2) a fast frequency modulation, typically a downsweep, 3) an elevated fundamental frequency, typically above 650 Hz (Vicario et al., 2001). The male distance call is learned from a tutor bird, similarly to how they learn song, and as such the call varies between individuals. There can be a large amount of variability in its composition based on the characteristics that are learned from the tutor, therefore this call varies greatly (Simpson & Vicario, 1990, 1991, Zann, 1985, 1990).

Lesioning brain regions that are critical for song learning (reviewed below) in male zebra finches causes their distance calls to become more female-like, and lose their male-typical characteristics (Simpson & Vicario, 1990). Some experimental manipulations can cause females to be able to learn and produce male-like distance calls, such as early life estrogen treatment (Simpson & Vicario, 1991). Early life exposure to high levels of estradiol caused a masculinization of vocal behaviour in female zebra finches: most treated females produced song-like vocalizations in adulthood, as well as being able to produce the male-typical aspects of the distance call (Simpson & Vicario, 1991). Therefore, in addition to learning, the correct hormones must be at play for males to produce their male-typical distance call, and this learning can occur if the brain is masculinized early in life. Female long-calls are mostly innate, not requiring any learning from a tutor bird.

1.4 Neural basis of birdsong and why calls have been overlooked when studying behavioural neurobiology

1.4.1 Song-control system

Birdsong is controlled by a series of interconnected brain nuclei and pathways called the song-control system (Nottebohm, 2005; Nottebohm & Arnold, 1976; Nottebohm, Stokes, & Leonard, 1976; Figure 1-7). This system is composed of two

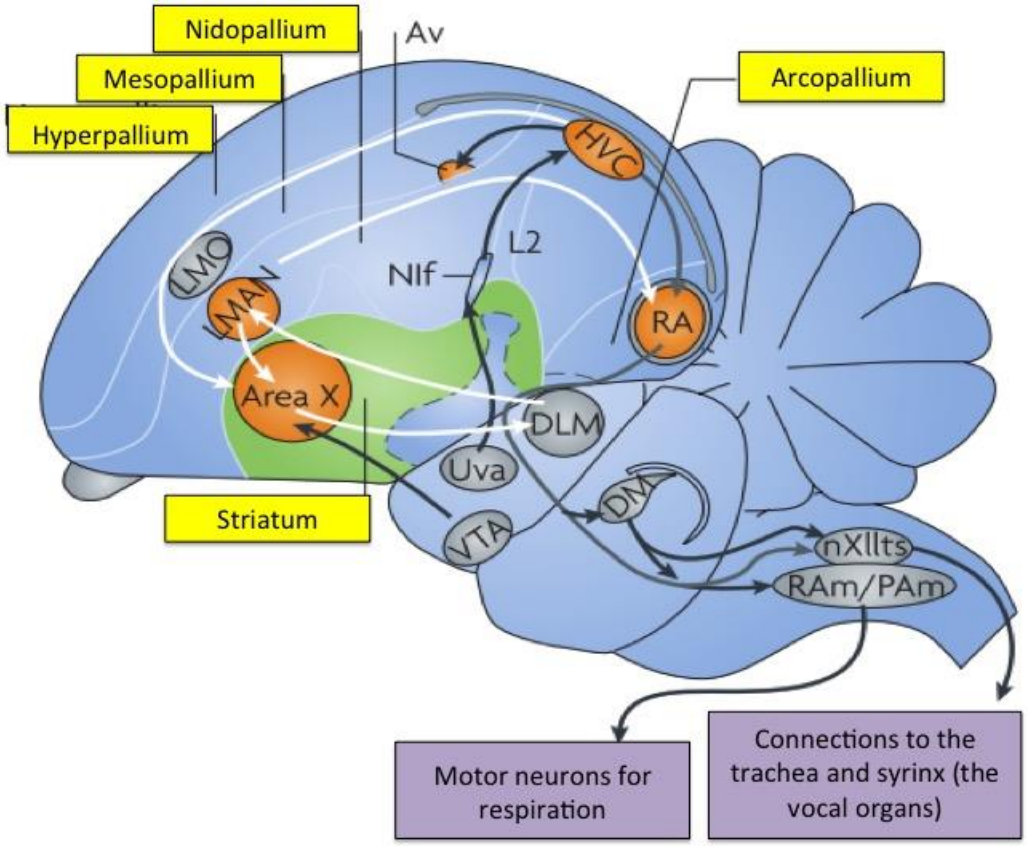

Figure 1- 7 Diagram depicting the parasagittal view of the song-control system of the songbird brain. Songbirds have a large variety of interconnected nuclei, divided into two pathways: the anterior forebrain pathway, depicted with white arrows, and the motor pathway, depicted with grey arrows. HVC, letter based name; Av, avalanche; LMO, lateral oval nucleus of the mesopallium; LMAN, lateral magnocellular nucleus of the anterior nidopallium; X, area X; NIf, interfacial nucleus of the nidopallium; RA, robust nucleus of the arcopallium; DLM, dorsal lateral nucleus of the medial thalamus; DM, dorsal medial nucleus of the thalamus; nXIIts, tracheosyringeal portion of the nucleus hypoglossus; RAm, nucleus retroambigualis; PAm, nucleus para-ambiguus; rVRG, rostro-ventral respiratory

group; Uva, nucleus uvaeformis; VTA, ventral tegmental area. The yellow boxes depict the different subdivisions of the songbird brain, whereas the purple boxes show where the projections go to outside the brain. Image is adapted from Bolhuis et al., 2010.

pathways: the anterior forebrain pathway and the descending motor pathway (Brenowitz, Margoliash, & Nordeen, 1997; Margoliash, 1997). The motor pathway is responsible for song production. HVC (not an acronym, used as a proper name) sends efferent projections to the robust nucleus of the arcopallium (RA), which projects to the dorsomedial nucleus of the midbrain nucleus intercollicularis (DM), that finally innervates the tracheosyringeal portion of the hypoglossal nucleus (nXIIts), as well respiratory control regions within the brainstem, in order to control the bird's vocal organ, the syrinx, during singing behaviour (Bolhuis & Gahr, 2006; Brenowitz et al., 1997; Margoliash, 1997; Nottebohm, 2005). This process occurs in a sequence and hierarchically: HVC encodes the higher-order song structure compared to RA, and HVC neurons will fire hundreds of milliseconds earlier than RA neurons prior to song onset (Yu & Margoliash, 1996). Early lesion studies were the first to demonstrate the importance of HVC and RA in song production (Nottebohm et al., 1976; Simpson & Vicario, 1990). Canaries (*Serinus canaria*) were subjected to bilateral HVC lesions, and singing behaviour was completely abolished; however, the birds would still posture as if they were singing. RA lesions did not have such effects: song was only detrimentally affected, but not completely abolished (Simpson & Vicario, 1990).

The anterior forebrain pathway is responsible for song learning, modification and maintenance and also begins with HVC. HVC connects to Area X, then to the nucleus dorsolateralis anterior pars medialis (DLM), to the lateral magnocellular nucleus of the anterior nidopallium (LMAN) and finally projecting to RA. Lesions to LMAN and area X

in juvenile zebra finches negatively affects song acquisition, but has little to no effect on song production and maintenance when conducted on adult zebra finches (Bottjer, Miesner, & Arnold, 1984; Sohrabji, Nordeen, & Nordeen, 1990). Within LMAN and area X there are neurons that are highly responsive to song-selective information, in particular a bird's own song, which allows for the auditory feedback necessary for normal song development (Doupe, 1997; Doupe & Konishi, 1991).

Although not part of the song-control system, there are auditory projections to the song-control system. HVC receives inputs from the nucleus interfacialis of the nidopallium, NIf, which is considered one of the main auditory inputs to HVC (Amador & Margoliash, 2011; Lewandowski, Vyssotski, Hahnloser, & Schmidt, 2013). HVC also receives inputs from the thalamic nucleus uvaeformis (Uva) and from auditory forebrain nuclei (caudomedial mesopallium, CMM; caudomedial nidopallium, NCM), which is necessary for the recognition and processing of song (Bolhuis & Gahr, 2006; Vates, Broome, Mello, & Nottebohm, 1996; Figure 1-8). Because HVC receives high-order auditory input, and organizes complex motor output, it can be thought of as analogous to association cortex in mammals.

1.4.2 Auditory Telencephalon

The auditory system in songbirds interacts with the song-control system in some respects and follows an ascending pathway similar the auditory system of mammals (see Figure 1-8). Auditory information travels from the cochlea to the auditory branch of the

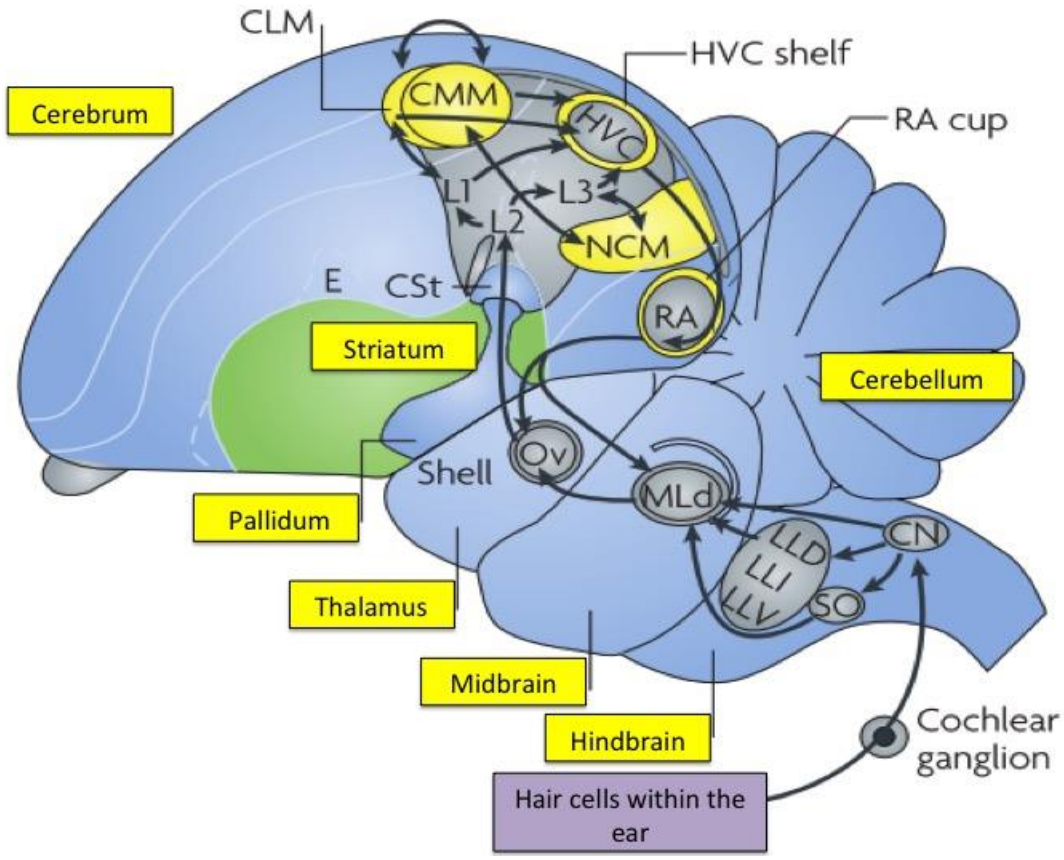

Figure 1- 8 Diagram depicting the parasaggital view of the auditory system of the songbird brain. Brain regions that show increased activation when the bird hears song are represented in yellow. CLM, caudal lateral mesopallium; CMM, caudomedial mesopallium; HVC, proper name; L1, L2, L3, subdivisions of Field L; NCM, caudomedial nidopallium; E, entopallium; CSt, caudal striatum; RA, robust nucleus of the arcopallium; Ov, ovoidalis; MLd, dorsal lateral nucleus of the mesencephalon; LLD, lateral lemniscus, dorsal nucleus; LLI, lateral lemniscus, intermediate nucleus; LLV, lateral lemniscus, ventral nucleus; SO, superior olive; CN, cochlear nucleus. The yellow boxes depict the different subdivisions of the songbird brain, whereas the purple box shows where the sensory information is coming from. Image is adapted from Bolhuis et al., 2010.

VIII cranial nerve, and then ascends to the brain through the dorsal lateral nucleus of the mesencephalon (MLd), then to the nucleus ovoidalis (Ov), then to the recipient zone of the telencephalon called Field L2, which is a dense granular cell layer that reciprocally projects to L1 and L3. Field L is thought to be homologous to primary auditory cortex of mammals. All of field L sends projections to the caudomedial nidopallium (NCM), the

caudomedial mesopallium (CMM), the caudolateral mesopallium (CML) and the caudal striatum (CSt). CLM reciprocally projects to the different components of Field L as well as to CMM. NCM also reciprocally projects to CMM, and Field L3 sends projections to NCM. NCM, CMM and CLM are considered secondary auditory cortical regions because they do not receive direct auditory input but are involved in the perceptual processing and discrimination of complex auditory stimuli like song or other vocalizations, as well as being able to process information in order to perform an associative learning task involving auditory cues (Amador & Margoliash, 2011; Bolhuis et al., 2010; Catchpole & Slater, 2008; Mello & Clayton, 1994).

Understanding how the song-control system works and how the auditory regions function is crucial in order to understand how the song-control system may be involved in the perception and production of learned calls. In fact, we know that lesioning HVC, RA and the tracheosyringeal nerves have a strong negative effect on song production as well as a strong negative effect on the defining characteristics of the male long-call, highlighting the importance of the song-control system in learned call production and also possibly innate call production (Simpson & Vicario, 1990; Ter Maat et al., 2014; Urbano, Aston, & Cooper, 2016). The neural processes underlying perception of learned calls are understudied (Avey, Kanyo, Irwin, & Sturdy, 2008; Eda-Fujiwara, Satoh, Bolhuis, & Kimura, 2003; Gobes et al., 2009; Roach, Lockyer, Yousef, Mennill, & Phillmore, 2016). The neural control of call production is even less studied in many species, including the black-capped chickadee. Budgerigars (a non-songbird species that also demonstrates vocal learning) show more neural perceptual activation in the auditory region NCM to more complex songs compared to simpler songs (Eda-Fujiwara et al.,

2003). Although this effect has been observed following song playback, if the results are primarily based on the acoustic complexity of the vocalization (defined as a vocalization with more notes, more rapid frequency modulations and larger frequency ranges) this may be applicable to learned calls as well. As noted above, the majority of research on the neurobiology of vocal production and perception has focused on songs and ignored calls; below I discuss why this is the case.

1.4.3 Calls have been ignored as a potential means of studying behavioural neurobiology

Birdsong is an elaborate behaviour. This vocalization is often complex, and it is performed in a conspicuous way typically to attract mates, and it is therefore unsurprising that research in behavioural neurobiology has primarily focused on these types of vocalizations (Catchpole & Slater, 2008; Kroodsma & Miller, 1996; Marler, 2004). Calls are much more variable, which in fact may make them harder to study (Marler, 2004). Bird calls are used in a variety of contexts: remaining in contact with the members of one's group, announcing the location of a food source, announcing the presence of a predator and indicating to parents to feed them (Beecher, 1982; Beecher et al., 1981; Leonard et al., 1997; Ligout et al., 2016; Medvin & Beecher, 1986; Rowley, 1980).

Part of the problem that has plagued behavioural neurobiology is the enormous variability in calls, not only with regard to their function, but also with regard to their acoustic structure, which varies from very simple to very complex. For a long time, calls were believed to be innate and not under the control of underlying neural structures that were devoted to the learning and production of song. However we now know that calls can be innate, learned, or partially learned (for review see Marler & Slabbekoorn, 2004;

Vicario, Raksin, Naqvi, Thande, & Simpson, 2002). With regards to learning, if the calls were believed to be innate, then the genetic basis of calls would have to be investigated; whereas if they are learned, the song-control system would be the ideal candidate for investigation. With more recent studies we know that this is in fact the case, that birds can have calls that are learned, partially learned, or innate, especially for black-capped chickadees and zebra finches (Baker et al., 2003, 2000; Baker & Gammon, 2008; Clemmons & Howitz, 1990; Ficken et al., 1985; Ficken & Weise, 1984; Ficken et al., 1987; Guillette et al., 2011; Hughes et al., 1998; Simpson & Vicario, 1990; Vicario et al., 2001; Zann, 1984).

Only recently has there been more investigation into call learning. In particular, the FoxP2 gene has been found to play a similar role in call learning as it does in song learning (Hara et al., 2015; Sewall, Young, & Wright, 2016; Whitney et al., 2014). There has also been evidence that some unlearned calls are controlled by some of the regions within the song-control system (Ter Maat et al., 2014). This emerging understanding that the song-control system also subserves call production provides the context for my thesis, which investigates the role that HVC, as well as other song-control nuclei, play in the production and perception of calls in the black-capped chickadee and the zebra finch (species that can learn calls as well as song).

1.5 Immediate-early genes and their use

In order to measure changes in activation within the brain we can use protein products of immediate-early genes (IEGs), which can be labeled and quantified using immunohistochemistry. The main IEG that has been used to investigate neuronal activation in avian brains is ZENK. ZENK is from the zinc finger family, and is an

acronym of four gene names of which it is the avian homologue: zif268, EGR-1, NGFI-A and krox24 (Avey et al., 2008; Avey et al., 2014; Brauth, Liang, Roberts, Scott, & Quinlan, 2002; Duffy, Bentley, & Ball, 1999; Leitner, Voigt, Metzdorf, & Catchpole, 2005; Mello, Vicario, & Clayton, 1992; Mello & Ribeiro, 1998; Phillmore, Bloomfield, & Weisman, 2003; Phillmore, Veysey, & Roach, 2011; Roach et al., 2016; Whitney, Soderstrom, & Johnson, 2000). ZENK is used as a short-term marker of brain activation, because within hours of a stimulus exposure, the protein products of the genes are produced and then degraded in active neurons (Cole, Saffen, Baraban, & Worley, 1989; Guzowski, Setlow, Wagner, & McGaugh, 2001; Mokin & Keifer, 2005; Thiriet, Zwiller, & Ali, 2001).

ZENK is a gene that encodes a nuclear transcription factor protein, ZENK, which is rapidly and transiently induced following exposure to extracellular stimuli. ZENK protein binds to DNA and activates transcription of target genes, and produces protein products that are required for cell division and differentiation. ZENK is not produced in all neuron types and populations, but cells expressing the ZENK protein in their nuclei are considered active, as in they are consistently being depolarized (Cole et al., 1989; Guzowski et al., 2001; Mokin & Keifer, 2005; Thiriet et al., 2001). ZENK is part of a molecular regulatory cascade of events, which begins with the activation of *N*-methyl-D-aspartate (NMDA-type) glutamatergic receptor activation, which leads to an intracellular influx of calcium (CA^{2+}). This influx of CA^{2+} leads to biochemical events which in turn lead to the induction of ZENK transcription and translation (Mello, 2002; Pinaud & Tremere, 2006). Cells then synthesizing ZENK protein during the presentation of

external stimulus can be quantified and measured as active. The number of active cells in a given area can be measured and will account for the area that is sampled.

Numerous studies have used ZENK to examine neuronal activation in the auditory regions in response to song and calls (Avey et al., 2008; Avey et al., 2014; Brauth, Liang, Roberts, Scott, & Quinlan, 2002; Duffy, Bentley, & Ball, 1999; Leitner, Voigt, Metzdorf, & Catchpole, 2005; Mello, Vicario, & Clayton, 1992; Mello & Ribeiro, 1998; Phillmore, Bloomfield, & Weisman, 2003; Phillmore, Veysey, & Roach, 2011; Roach et al., 2016, 2011; Whitney, Soderstrom, & Johnson, 2000). In addition, because ZENK immunoreactivity (ZENK-ir) can be driven by motor activity as well as auditory experience, ZENK has been used as a means of identifying structures involved in singing behaviour, even in non-oscine species, as well as identifying relationships between the song-control system and the auditory forebrain regions (Jarvis et al., 2000; Liu, Wada, Jarvis, & Nottebohm, 2013; Vates et al., 1996). Songbirds tend to show more observable neuronal activation in auditory regions NCM and CMM in response to more complex songs, as well as better quality songs, compared to simpler songs (Gentner, Hulse, Duffy, & Ball, 2001; Leitner et al., 2005). However black-capped chickadees have shown conflicting results in terms of ZENK-ir in the auditory regions (Avey et al., 2008; Phillmore et al., 2003). Phillmore and colleagues (2003) found that black-capped chickadees showed more neuronal activation in the auditory regions for the *fee-bee* song compared to the *chick-a-dee* call. In contrast, Avey and colleagues (2008) found that chickadees showed more activation in the auditory regions for *chick-a-dee* call compared to the *fee-bee* song. Therefore, it is unclear what aspects of the vocalizations chickadees are attending to, and whether ZENK response in CMM and NCM reflect the meaning of

the vocalization, the acoustic complexity of it, or whether or not there is a learned component (Hernandez et al. 2008; Gentner et al., 2001).

1.6 Thesis objectives

The overall objective of this thesis was to further understand the neural mechanisms of bird calls, both in production and neural basis of perception. My primary goals were to (1) understand the involvement of the song-control system in the production of calls, (2) understand how bird calls are perceived in the brain, (3) and if the song-control system is involved in the neural basis of perception of bird calls. For my experiments I used two different species: the black-capped chickadee (*Poecile atricapillus*) and the zebra finch (*Taeniopygia guttata*). I chose these two species for different reasons. The black-capped chickadee produces learned vocalizations throughout the year, and these vocalizations are produced by both sexes (Ficken et al., 1978). Also unlike many songbirds, their song is not the most complex vocalization they produce, which allows me to tease apart whether acoustic complexity (defined as a vocalization with more notes, more rapid frequency modulations and larger frequency ranges) or the amount of learning required to produce the vocalization is driving the neural basis of perception of bird calls. Chickadee calls are also partially learned, which leads to the possibility that the song-control system is involved in their production, and is why for the majority of my studies I used the black-capped chickadee. I also used the zebra finch because it has a well-established brain atlas, which facilitated successful lesion locations, in order to examine the involvement of HVC in the neural basis of perception of their learned call. Although zebra finches are sexually dimorphic in singing, I was able to

examine differences in the perception of a learned call in males and females, and examine how this changes in males when they no longer possess a functional HVC.

1.6.1 The song-control system and call production

The song-control system is involved in the learning and production of song, however very little research has been done to examine its involvement in call production (Roach et al., 2016; Ter Maat et al., 2014). In Chapter 2, I examined the involvement of the song-control system in the production of different calls. I accomplished this by examining motor-driven IEG expression in two song-control nuclei, HVC and RA, when chickadees produced their *fee-bee* song, *chick-a-dee*, *gargle*, and *tseet* calls. I predicted that chickadees producing the *fee-bee* song would show the most activation in both HVC and RA, followed by birds producing the *gargle* and *chick-a-dee* calls, which would show similar levels of ZENK immunoreactivity (-ir). Finally I predicted that the *tseet* group would show little ZENK-ir, and birds who were silent would show little to no activation. In Chapter 3, I examined the importance of the song-control system in the production of calls. I accomplished this by lesioning HVC in black-capped chickadees and examining the subsequent effects on their *gargle* and *chick-a-dee* calls. Because the *gargle* and *chick-a-dee* calls show learned components, I hypothesized that by lesioning HVC I would detrimentally affect the *gargle* call, and the B and C notes of the *chick-a-dee* call (Baker et al., 2003, 2000; Bloomfield et al., 2008; Clemmons & Howitz, 1990; Freeberg, 2012; Hughes et al., 1998).

1.6.2 Neural basis of perception of bird calls

Neural basis of perception of song is typically dependent on song complexity, as well as song quality (Gentner et al., 2001; Leitner et al., 2005). Therefore it seems likely

that neural basis of perception could be due to acoustic complexity, meaning or learning of the vocalization. I could tease apart these possibilities by using black-capped chickadees because their vocalizations vary in the amount of learning they require as well as their acoustic complexity. In Chapter 4, I examined the neural basis of perception of song and calls in the auditory regions of the songbird brain. I accomplished this by playing back *fee-bee* songs, *chick-a-dee* calls, *gargle* calls, pink-noise or silence to black-capped chickadees and then examined the neuronal activation in the auditory regions NCM and CMM. I predicted that if the activity of these regions was modulated by call complexity, I would see the highest amount of ZENK-ir in CMM and NCM for the *gargle* call, followed by the *chick-a-dee* call and then the *fee-bee* song.

1.6.3 The song-control system and the neural basis of perception of bird calls

To my knowledge, no studies have investigated the probable role of the song-control system in the neural basis of perception of call processing in auditory regions. Only one study has shown that a song-control nucleus is involved in the neural basis of perception of calls (Vicario et al., 2001), and it was RA, a structure typically only associated with the production of vocalizations. The involvement of HVC in call perception is still unclear, which is why I used zebra finches to examine this question. In Chapter 5, I lesioned HVC in zebra finches and examined the activation of auditory regions NCM and CMM in response to female and male long-calls. I used zebra finches because their responses to female and male long-calls are well studied, both behaviourally and within the brain (Gobes et al., 2009; Simpson & Vicario, 1990; Vicario et al., 2001, 2002; Vicario, 2004; Vicario et al., 2001). I predicted that HVC lesioned

males and intact females would have similar levels of ZENK-immunoreactive (-ir) expression in response to male and female long-calls. Based on previous findings, I predicted that the HVC lesioned males and females would show increased ZENK-ir expression in NCM and CMM to the female long-call, whereas males would not (Gobes et al., 2009). Overall, my studies aimed to showcase the involvement of the song-control system in call production and neural basis of perception in the black-capped chickadee and the zebra finch.

1.7 References

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Chapter 2

2 Motor-driven gene expression in the song-control system of the black-capped chickadee

2.1 Introduction

Imitative vocal learning of simpler vocalizations, or "calls" can be observed in songbirds, elephants, bats, parrots, whales, seals and primates (Doupe & Kuhl, 1999). Although deemed simpler than more complex sounds such as birdsong, calls are used in a large variety of social contexts, such as maintaining contact during foraging, displaying aggressive behaviours, announcing the presence of a predator or of a food source. These calls are therefore crucial to the animal's survival (Tyack, 2008). Black-capped chickadees not only learn and produce their *fee-bee* song (Kroodsma, Albano, Houlihan, & Wells, 1995; Shackleton & Ratcliffe, 1993), but also produce a variety of other calls that vary in complexity: the *gargle*, the *chick-a-dee*, and the *tseet* calls (for complete repertoire see Ficken, Ficken, & Witkin, 1978). These calls are used to display aggression, alert others of the presence of a predator and maintain contact with members of a flock and are therefore crucial for individual chickadees' survival (Otter, 2007).

The *chick-a-dee* call is one of the more extensively studied calls that the black-capped chickadee produces. The *chick-a-dee* call is composed of multiple note types (A, B, C, and D notes, see Figure 2-1) and is at least partially learned (Hughes, Nowicki, &

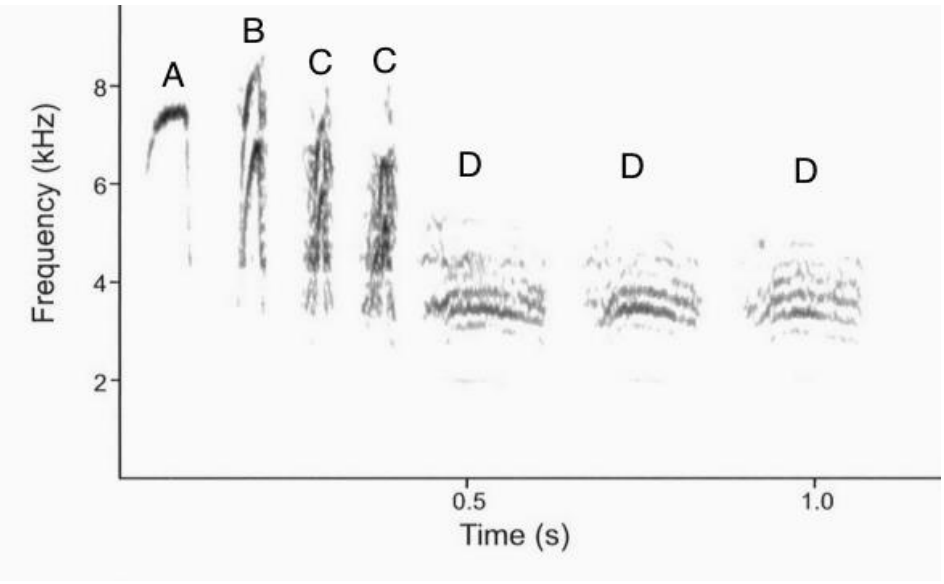

Figure 2- 1 Spectrogram of the black-capped chickadee *chick-a-dee* call. This figure depicts the four different note types which comprise the *chick-a-dee* call; A, B, C and D notes. Figure adapted from Charrier et al., 2004b.

Lohr, 1998). Chickadees raised in complete isolation still produced wild-type sounding A and D notes, however the B and C notes almost completely disappeared - very few B and C notes are produced by birds raised in isolation. Exposure to wild type *chick-a-dee* calls is crucial for the normal development of those B and C notes, indicating that the call may be both partially learned and partially innate.

The *gargle* call is also not entirely innate as previously believed, but shows geographic variation in acoustic structure (Baker, Howard, & Sweet, 2000). Chickadees recorded over an 8.4 km geographical region show geographic variation in their *gargle* calls. The birds produce *gargle* calls that are unique to a particular geographic location, and share some features of the *gargle* calls across some or all areas. Therefore, at a small geographic distance there are differences in the *gargle* call, which may be due to the birds learning the *gargle* call.

There has been very little research conducted on the *tseet* call, however the *tseet* call has been found to contain relevant information of the caller's species, sex and individual identity (Guillette et al., 2010). The *tseet* call could be used to distinguish between black-capped and mountain chickadees. These calls may be innate, and be genetically coded for within the species, but because they also differ between individuals they could also be learned (Guillette et al., 2010). Therefore the *gargle, chick-a-dee* and *tseet* calls are good candidates to examine the neural basis of call production.

The underlying neural mechanisms for learning and producing birdsong have been extensively studied (Brenowitz et al., 1997; Nottebohm, Stokes, & Leonard, 1976; see reviews Nottebohm, 2005; Schmidt, 2009). However very little research has focused on the underlying neural mechanisms of calling behaviour in songbirds (Brauth, Liang, Roberts, Scott, & Quinlan, 2002; Marler, 2004; Sewall et al., 2016; Ter Maat, Trost, Sagunsky, Seltmann, & Gahr, 2014). Due to the mounting evidence that some bird calls are in fact learned and not innate as previously believed, it is crucial to understand if the song-control system is involved in the production of calls as well as song (Catchpole & Slater, 2008; Kroodsma & Miller, 1996).

The nuclei of the song-control system in temperate-zone songbirds typically show seasonal variation in their size (Nottebohm, 1981; Kirn, Clower, Kroodsma & DeVoogd, 1989; Brenowitz, Nalls, Wingfield, & Kroodsma, 1991; Smith et al., 1995; Smith, 1996; Brenowitz, Baptista, Lent, & Wingfield, 1998; Ball et al., 2004). During the breeding season (typically the springtime), there is an increase in singing behaviour that is associated with an increase in size of the song-control nuclei. This variation has also been shown in Parids, specifically the blue tit (Caro, Lambrechts, Balthazart, 2005). However

black-capped chickadees, who are also Parids, do not show these seasonal variations (Phillmore, Hoshooley, Sherry, & Macdougall-Shackleton, 2006; Smulders et al., 2006). It is plausible that the song-control system may be controlling more than just the *fee-bee* song in black-capped chickadees (Smulders et al., 2006). Although there is an increase in *fee-bee* songs during the springtime, the song-control nuclei may be maintained year-round to control the production of their other vocalizations (i.e., the *gargle*, *chick-a-dee,* and *tseet* calls). The song-control nuclei are therefore the perfect candidates in which to investigate the underlying neural mechanisms of call production in chickadees. Neural activation can be measured by using the immediate-early gene ZENK. Large increases in expression of the immediate-early gene ZENK in HVC, RA and area X have been previously associated with singing behaviour in canaries (*Serinus canaria*; Jarvis & Nottebohm, 1997). This motor-driven gene expression is also independent of auditory feedback, as it occurs even in singing deaf birds. ZENK immunoreactivity (ZENK-ir) is also quantitatively proportional in its expression to the amount of singing that occurs (Jarvis et al., 2000)

The objective of this study was to determine the role that HVC and the robust nucleus of the arcopallium (RA) play in the production of the *gargle*, *chick-a-dee* and *tseet* calls in chickadees. I predicted that if HVC controls the production of learned calls then it should exhibit increased ZENK-ir following calling. I captured black-capped chickadees and put them in social and acoustic isolation from one another before exposing them to various stimuli in order to elicit the *fee-bee* song, *gargle*, *chick-a-dee* and *tseet* calls. Birds were divided into treatment groups based on which vocalizations were produced during stimulus presentation (i.e., *fee-bee* song group, *gargle* call group,

chick-a-dee call group, *tseet* call group, silent control group). Following the production of the vocalizations, the birds were euthanized and the brains were collected for processing. I used the immediate-early gene ZENK (an acronym for Zif-268, Egr-1, NGFI-A, and Krox-24) to quantify the amount of neuronal activation in HVC and RA during the different call productions; an established technique (Jarvis & Nottebohm, 1997). There are a variety of studies that showcase the involvement of HVC and RA in singing behaviour in songbirds, and also show that HVC is crucial for the production of the male long-call in zebra finches (Catchpole & Slater, 2008; Kroodsma & Miller, 1996; Marler, 2004; Simpson & Vicario, 1990). Therefore, learning may be the crucial component responsible for the involvement of HVC in vocal production. The more learning that occurs for a particular vocalization, the more HVC may be involved. I predicted that chickadees producing the *fee-bee* song would show the most activation in both HVC and RA, because we have the most evidence that this vocalizations is learned, followed by birds producing the *gargle* and *chick-a-dee* calls, which would show similar levels of ZENK immunoreactive (-ir) expression. Finally I predicted that the *tseet* group would show little ZENK-ir expression, because there is the least evidence that this call is learned, and birds who were silent would show little to no activation.

2.2 Methods

2.2.1 Subjects and housing

In late 2012 and early 2013, I captured a total of 25 adult black-capped chickadees (*Poecile atricapillus*) on the University of Western Ontario campus, London, Ontario (43˚01’ N, 81˚27’ W). I identified birds as either male (n = 18) or female (n = 7) based on body mass and wing chord measurements, which I later confirmed by

examining the gonads post-mortem. Birds were initially group-housed (range: 3-4 birds per cage) in an outdoor aviary. Birds had ad libitum access to food (Mazuri small-bird maintenance diet mixed with black-oil sunflower seeds) and water; their diet was also supplemented with mealworms (2 worms per individual per day).

I used a variety of methods to elicit different type of vocalizations from the birds. Birds were exposed to different stimuli (i.e., novel live chickadee, stuffed saw-whet owl (*Aegolius acadicus)*, mirror, or sunlight) and I monitored their behavioural and vocal responses. The *chick-a-dee* call group (*n* = 5) produced primarily *chick-a-dee* and *tseet* calls. The *fee-bee* song group (*n* = 4) produced primarily *fee-bee* songs and *tseet* calls. The *gargle* call group (n = 5) produced primarily the *gargle* and *tseet* call. The *tseet* call group (*n* = 5) produced primarily the *tseet* call, and the control group (*n* = 2) remained relatively silent. One bird from those caught was used to practice the immunohistochemistry technique.

2.2.2 Behavioural recordings

I took birds in the *chick-a-dee* call group from their home cage and placed them into a wire cage lined with newspaper in a modified audiometric testing booth (width 91cm X height 172cm X depth 71cm) for 24-48 hours, where the photoperiod was matched to ambient outdoor conditions. Following the isolation period, I removed the food and water dishes from the cage and exposed the birds to one of two possible stimuli placed within the modified audiometric testing booth but outside of the wire cage: a mirror or a taxidermy saw-whet owl in order to elicit the *chick-a-dee* call, which is an indicator of mild alarm (Ficken, Ficken, & Witkin, 1978). I recorded the birds using a Marantz PMD 671 recorder attached to a Sennheiser microphone and a JVC handheld

camcorder (GZ-MS120) for a period of 15-min, quantified the number and variety of calls, and confirmed the counts when listening and viewing recordings of the session. I removed the stimulus, the food cup was returned, and the bird was left in isolation within the chamber for an hour before it was euthanized by transcardial perfusion and the brain collected (see below).

I conducted the same experimental procedures as described above for the birds in the *gargle* call group and *tseet* call groups except that the stimulus was an unfamiliar live chickadee (captured from a different location). Both chickadees were put into the same wire cage inside the audiometric testing booth, and the black-oil sunflower seed cup was not removed but placed directly between the two perches inside the cage to incite an aggressive encounter between the birds. Immediately following the 15-min exposure, the birds were separated and returned to isolation for an additional hour and the video recording was examined to determine which bird was primarily producing *gargle* calls and which one was producing mostly *tseet* calls. One of the birds was then euthanized by transcardial perfusion and the brain collected. In the first session the bird producing the gargle calls was euthanized, whereas the following exposure the bird producing the tseet calls was euthanized, and this alternated until all the brains were acquired for each experimental condition. The birds in the silent control group were not presented a stimulus, but all other parameters remained the same as those for the birds in the *gargle* and *tseet* call groups. Birds in the *fee-bee* song group were left in the outdoor aviaries and recorded only using the Marantz PMD 671 recorder attached to a Sennheiser microphone during their pre-dawn chorus (range: 5:15 – 5:45 a.m.). They were not video recorded due to dark conditions during sunrise and possible interference from the camera during

the dawn chorus. When I heard the first *fee-bee* song, I identified the singer and set a timer for 15-min. I recorded the number of *fee-bee* songs produced during that time, and later confirmed when listening to the recording. At the end of the 15-min, I caught the singer and placed them in isolation for an hour prior to euthanizing them and collecting the brain (see below).

For birds in all of the above groups, following the hour of isolation I anesthetized birds using isoflurane. Following deep anesthesia, birds were euthanized by transcardial perfusion with 0.1M phosphate buffered saline (PBS) followed by buffered 4% paraformaldehyde. I quickly removed the brain from the skull and placed it in 4% paraformaldehyde (~24 h) and then in 30% sucrose (~36 h) at 4 ˚C. Brains were then frozen on crushed dry ice and then stored at -80 ˚C.

2.2.3 Call quantification

Using RavenPro 1.4 (Bioacoustics Research Program, 2011), and plotting the spectrogram of each session, I quantified the number of songs and calls produced in each recording for each bird tested. The vocalizations were identified as *fee-bee* songs, *gargle, chick-a-dee* or *tseet* calls, and the number of vocalizations of each different type was recorded. For the recordings of the *gargle* and *tseet* calls I used the video recordings. I determined which bird was making each vocalization in the trials where 2 chickadees were present in the same cage. The chickadees were easily identifiable from one another due to different coloured leg bands on different individuals.

For the *chick-a-dee* call recordings, I quantified the total number of *chickadee* calls produced. However, because the length of the *chickadee* call can vary greatly due to

the number of repetitions of D notes produced per call, I also separated the chickadee calls into two components, the ABC complex and the D notes, and I quantified the number of D notes produced per call. The number of D notes increases the length of the call, and because ZENK-ir is correlated with the amount of behaviour it was an additional measure to be considered. Therefore I had a total number of *fee-bee* songs, as well as *gargle*, *chick-a-dee* (separated into ABC complex and D notes, and then combined into a total number of ABCD calls) and *tseet* calls.

2.2.4 Nissl histology

In order to identify brain structures I Nissl-stained sections with thionin. Using the cryostat, I sectioned brains into 40 µm coronal sections, and put every third series into 0.1 M PBS for Nissl histology, ZENK immunohistochemistry (see below), and a back-up series. The sections were washed and temporarily stored in 0.1 M PBS (pH 7.5). I mounted sections onto gelatin coated microscope slides, and let them air-dry overnight. Next sections were stained using thionin followed by serial dehydrations with increasing concentrations of ethanol, and cleared of lipids with an organic solvent (NeoClear, cat no. 65038-71; EMD Chemicals, Mississauga, Ontario, Canada). Finally slides were covered with coverslips using a mounting medium (Permount, cat no. SP15; Fisher Scientific) and allowed to dry in a fume hood for about 12 h.

2.2.5 ZENK immunohistochemistry

I ran immunohistochemistry in multiple runs counterbalanced across the different vocalization groups. I used an established immunohistochemistry protocol (Farrell, Neuert, Cui, & MacDougall-Shackleton, 2015; Hernandez & MacDougall-Shackleton, 2004; Maney, MacDougall-Shackleton, MacDougall-Shackleton, Ball, & Hahn, 2003;

McKenzie, Hernandez, & MacDougall-Shackleton, 2006; Schmidt, McCallum, MacDougall-Shackleton, & MacDougall-Shackleton, 2013). First, using the cryostat, I sliced brains into 40 µm coronal sections and temporarily stored them in 0.1M PBS. Every third section (i.e., 120 µm) was used to examine ZENK immunoreactivity (ZENK-ir). First, free-floating sections were thoroughly rinsed twice with 0.1M PBS, and then incubated with 0.5% H_2O_2 in PBS for 15 min to eliminate endogenous peroxidase activity. Sections were washed three times with 0.1 M PBS, and then incubated in 10% Normal Goat Serum (cat no. S-1000; Vector Laboratories, Burlingame, CA USA) in 0.1 M PBS containing 0.3% Triton X-100 (0.3% PBS/T) for 1 h. Sections were then incubated with primary antibody made in rabbit against Egr-1 (polyclonal, 1:4000, cat no. SC-189; Santa Cruz Biotechnology, Santa Cruz, CA USA) in 0.3% PBS/T for ~24 h at 4 ˚C. After rinsing three times with 0.1% PBS/T, sections were incubated with biotinylated goat anti-rabbit IgG secondary antibody (1:250 dilution) for 1 h at room temperature, followed by three rinses with 0.1% PBS/T. Sections were then incubated with avidin-biotin horseradish-peroxidase complex (VectaStain Elite ABC Kit, cat no. PK 6100; Vector Laboratories) at dilution 1:200 for 1 h, followed by two rinses with 0.1% PBS/T. The tissue sections' immunoreactivity was then visualized with 3, 3'-diaminobenzidine tetrahydrochloride (SigmaFAST DAB, cat no. D4418; Sigma). After thoroughly rinsing the sections with PBS, I mounted the sections onto gelatin coated microscope slides, and left them to dry overnight. Once dry, I put the slide through serial dehydrations with increasing concentrations of ethanol, and cleared lipids with an organic solvent (NeoClear, cat no. 65038-71; EMD Chemicals, Mississauga, Ontario, Canada). Finally, slides were covered with coverslips using a mounting medium (Permount, cat no. SP15;

Fisher Scientific) and allowed to dry in a fume hood for about 12 h. One brain was lost from the control group during the immunohistochemistry procedure due to poor staining.

2.2.6 ZENK quantification

ZENK-ir was quantified for two song-control nuclei: HVC and RA (see Figure 2-4 and 2-7) by using a Leica DM 5500B microscope coupled to a Leica 420C camera. I determined the locations of HVC and RA using the thionin Nissl-stained tissue. Next, the ZENK stained tissue from adjacent sections was used to capture images for further analysis. For each chickadee, 10 to 12 images were captured for HVC (~5-6 images/hemisphere), and four to eight images were captured for RA (~2-4 images/hemisphere). Images were first taken from the slice with the largest cross-sectional area of HVC or RA present in the slice. Subsequent images were taken from the few slices more rostral and more caudal from the largest point of the structure. The sections were selected such that the middle of the imaged sections contained the largest cross-section of song-control region. For HVC and RA, each image was taken such that the region of interest was located centrally in the image, and contained most or all of the structure. For each field of interest, z-stack images of 0.63 μm steps through the focal planes were collected through the 20× objective lens and were then compiled using a montage mode in Leica Application Suite software. This allowed for all of the ZENK-ir cells to be in focus within the same image. For each image, I traced the outline of the structure, and the area (mm^2) was determined. I counted the number of ZENK-ir cells following a semi-automated protocol using the ImageJ program (NIH). Briefly, images were opened in ImageJ and were automatically adjusted to gray scale, autocontrasted and

auto-thresholded. The threshold was adjusted in order to ensure that only immunoreactive

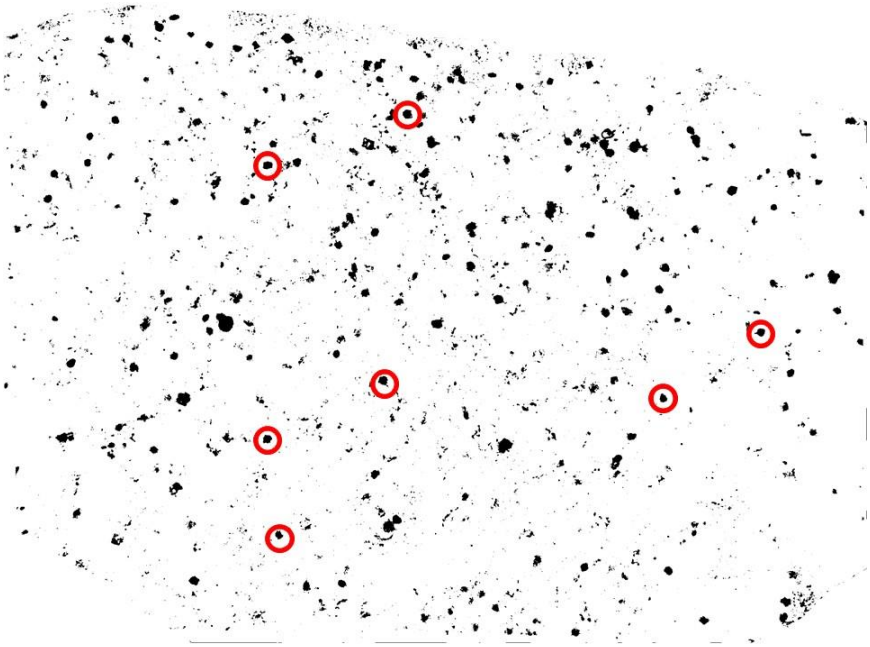

Figure 2- 2 Image depicts ZENK-ir cells in HVC after the image has been transformed to greyscale and autocontrasted. The red circles highlight examples of some of the cells that would be counted. Smaller objects were excluded from cell counts.

cells were highlighted. Minimum and maximum cell sizes were based on prior studies were used to exclude non-cell objects (9.07-27.21 µm) and a minimum sphericity of 0.65 was used in ImageJ during the cell counting procedures. The measurements for area (mm^2) and cell counts were entered in a spreadsheet and the number of cells/mm^2 was determined in order to control for any size differences in HVC across individual birds. I also had a blind observer who recaptured all images for HVC and RA, compiled and analyzed them using the same guidelines, and was blind to the treatment group of each subject to determine inter-rater reliability and to account for any biases in picture taking or processing.

2.2.7 Data and statistical analyses

Only one set of ZENK-ir cell counts and structure areas was used due to high reliability between observers (89%). Statistical analyses were carried out using IBM SPSS Statistics 24.0. The mean number of cells/mm^2 for each individual was compared among the right and left hemispheres using a paired t-test. No significant differences were found among hemispheres; therefore all analyses were conducted on the mean cell count pooled among hemispheres.

I first tested for correlations between the number of calls (i.e., *gargle*, *chick-a-dee*, *tseet* and *fee-bee*) and the number of ZENK-ir cells in HVC and RA. This analysis included birds from all groups pooled together, as in birds in each group often produced more than one type of call. For example, the *tseet* call was produced in all call groups.

Following the correlation analysis I tested whether the number of ZENK-ir cells in HVC and RA varied across the playback groups using a one-way ANOVA, with vocalization group as factor and sex as a covariate. Results were considered significant at $\alpha \leq 0.05$ level. Data are presented as mean ± SEM.

2.3 Results

2.3.1 HVC

Sex was found to be a non-significant covariate for ZENK-ir in HVC ($F(1,14) = 0.009$, $p = 0.926$) and was removed from the analyses. Across all birds number of *gargle* calls uttered was highly correlated with the number of ZENK-ir cells in HVC (r (18) = 0.669, $p = 0.001$), the more calls that were produced the more ZENK-ir cells were found in HVC (see Figure 2-3). No other vocalization showed a significant correlation to

ZENK-ir cells in HVC, and no vocalizations were correlated with one another (Table 2-1).

Table 2- 1 Correlation matrix depicting correlations between the call counts for the *chick-a-dee, gargle, fee-bee* and *tseet* groups with the number of ZENK-ir cells expressed in HVC.

		Chick-a-dee Call	*Gargle Call*	*Fee-bee Song*	*Tseet Call*	*ZENK-ir Cells HVC*
Chick-a-dee Call	*r*	1				
	p					
	n	21				
Gargle Call	*r*	-0.212	1			
	p	0.356				
	n	21	21			
Fee-bee Song	*r*	-0.125	-0.161	1		
	p	0.588	0.486			
	n	21	21	21		
Tseet Call	*r*	-0.263	0.223	-0.268	1	
	p	0.250	0.330	0.240		
	n	21	21	21	21	
ZENK-ir Cells HVC	*r*	-0.047	**-0.669***	-0.227	0.386	1
	p	0.843	0.001	0.336	0.093	
	n	20	20	20	20	20

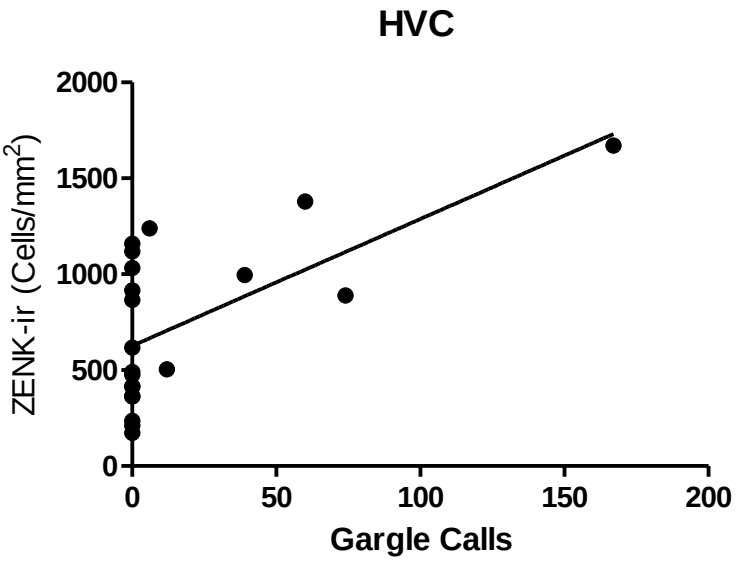

Figure 2- 3 Correlation between the number of *gargle* calls produced and the amount of ZENK-ir cells in HVC of adult black-capped chickadee for the *gargle* call only for all birds in all groups. The more *gargle* calls were produced, the more ZENK-ir activity there is in HVC.

For the different groups, there was a significant main effect of vocalization group, $F(4, 15) = 7.889$, $p = 0.001$. A Tukey post-hoc test revealed that the birds in the *gargle* group had significantly more ZENK-ir cells in HVC than birds in the *tseet* group ($p = 0.009$), the *fee-bee* group ($p = 0.006$), and the control group ($p = 0.003$) (Figure 2-4, 2-5). However the birds in the *gargle* group did not differ in ZENK-ir cells in HVC from the *chick-a-dee* group ($p = 0.129$). The number of ZENK-ir cells in HVC for *chick-a-dee* call group did not differ from any other group ($p > 0.05$). And the number of ZENK-ir cells in HVC of the *tseet*, control and *fee-bee* groups did not differ from one another ($p > 0.05$).

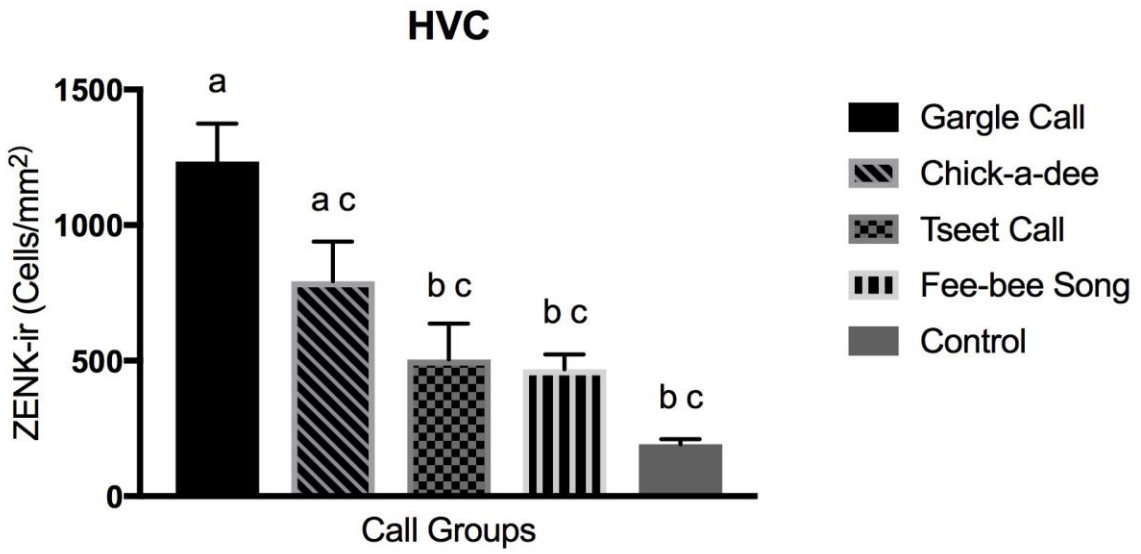

Figure 2- 4 Effect of vocalization type on the total number of ZENK-ir cells in HVC of adult black-capped chickadees. The birds in the *gargle* call group had more ZENK-ir cells in HVC than the *tseet* call, the *fee-bee* song and the control groups. The letters represent statistical differences between the groups; letters that share the same lower case letter did not significantly differ from each other.

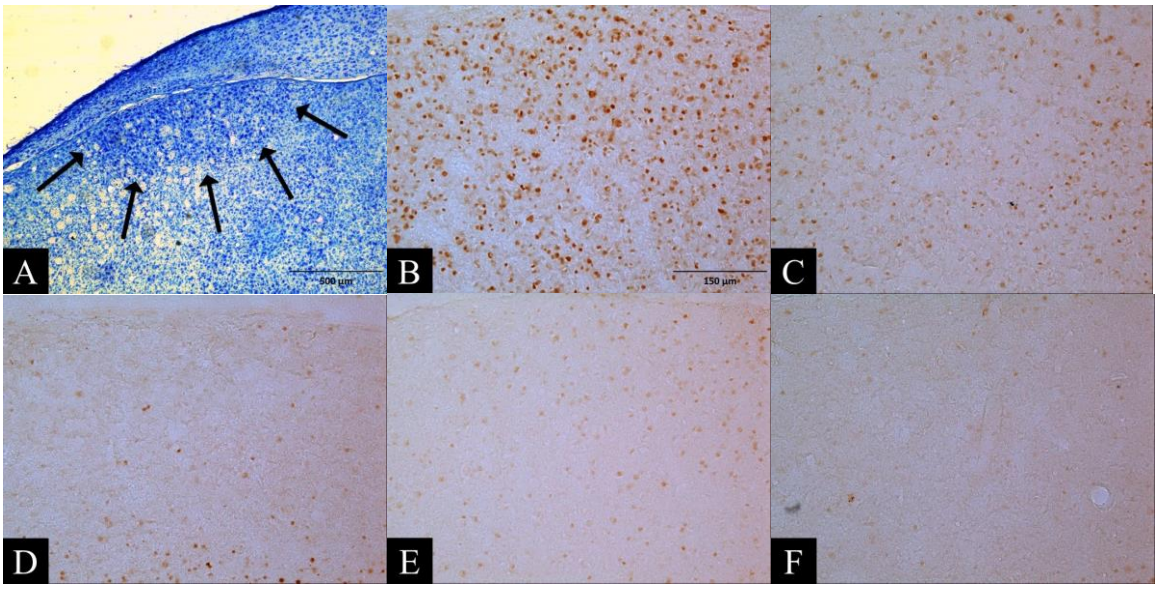

Figure 2- 5 Example ZENK immunoreactivity in HVC of black-capped chickadees in each of the five vocalization conditions. A) Sagittal section of Nissl stained HVC. B) ZENK immunoreactivity of black-capped chickadees producing *gargle* calls, C) *chick-a-dee* calls D) *tseet* calls and E) *fee-bee* songs. F) ZENK immunoreactivity of the silent black-capped chickadee control. Images B, C, D, E, F are all taken at the same magnification, and use the same scale. Anterior is up and caudal is to the left in all images.

2.3.2 RA

Sex was found to be a non-significant covariate for RA ($F(1,15) = 0.139$, $p = 0.714$) and was removed for the analyses. The number of *gargle* calls uttered was highly correlated with the number of ZENK-ir cells in RA (r (19) = 0.836, $p < 0.001$), the more calls that were produced the more ZENK-ir cells were observed in RA (see Figure 2-6). No other vocalization showed a significant correlation to ZENK-ir cells in RA, and no vocalizations were correlated with one another (Table 2-2).

Table 2- 2 Correlation matrix depicting correlations between the call counts for the *chick-a-dee, gargle, fee-bee* and *tseet* groups with the number of ZENK-ir cells expressed in RA.

		Chick-a-dee Call	*Gargle Call*	*Fee-bee Song*	*Tseet Call*	*ZENK-ir Cells HVC*
Chick-a-dee Call	*r*	1				
	p					
	n	21				
Gargle Call	*r*	-0.212	1			
	p	0.356				
	n	21	21			
Fee-bee Song	*r*	-0.125	-0.161	1		
	p	0.588	0.486			
	n	21	21	21		
Tseet Call	*r*	-0.263	0.223	-0.268	1	
	p	0.250	0.330	0.240		
	n	21	21	21	21	
ZENK-ir Cells HVC	*r*	-0.303	**-0.836***	-0.047	0.327	1
	p	0.181	<0.001	0.841	0.148	
	n	20	20	20	20	21

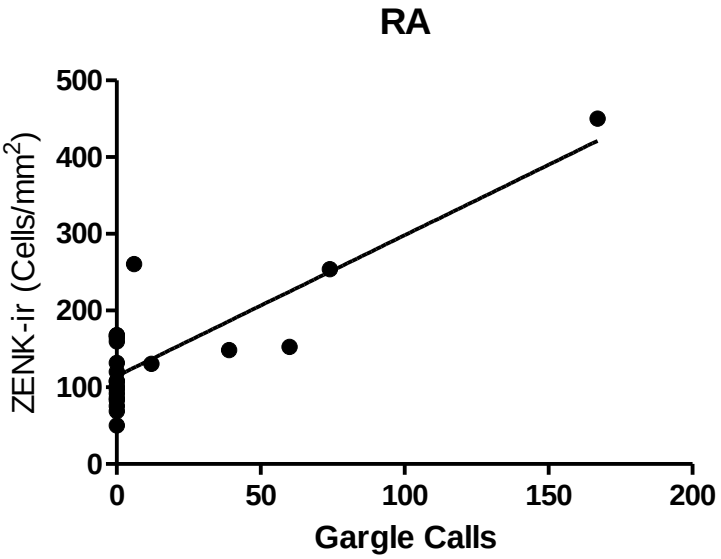

Figure 2- 6 Correlation between the number of *gargle* calls produced and the amount of ZENK-ir cells in RA of adult black-capped chickadee for the *gargle* call only for all birds in all groups. The more *gargle* calls were produced, the more ZENK-ir activity there is in HVC.

There was also a significant main effect of vocalization group, $F(4, 16) = 4.547$, $p = 0.012$. A Tukey post-hoc test revealed that the birds in the *gargle* group had significantly more ZENK-ir cells in RA than birds in the *chick-a-dee* group ($p = 0.013$) and the control group ($p = 0.044$) (Figure 2-7, 2-8). However, the birds in the *gargle* group did not differ in ZENK-ir cells in RA from the *tseet* group ($p = 0.097$) or the *fee-bee* group ($p = 0.082$). The number of ZENK-ir cells in RA for the *tseet* call and *fee-bee* song groups did not differ from any other group ($p > 0.05$). And the number of ZENK-ir cells in RA for the *chick-a-dee* call and control groups did not differ from one another ($p > 0.05$).

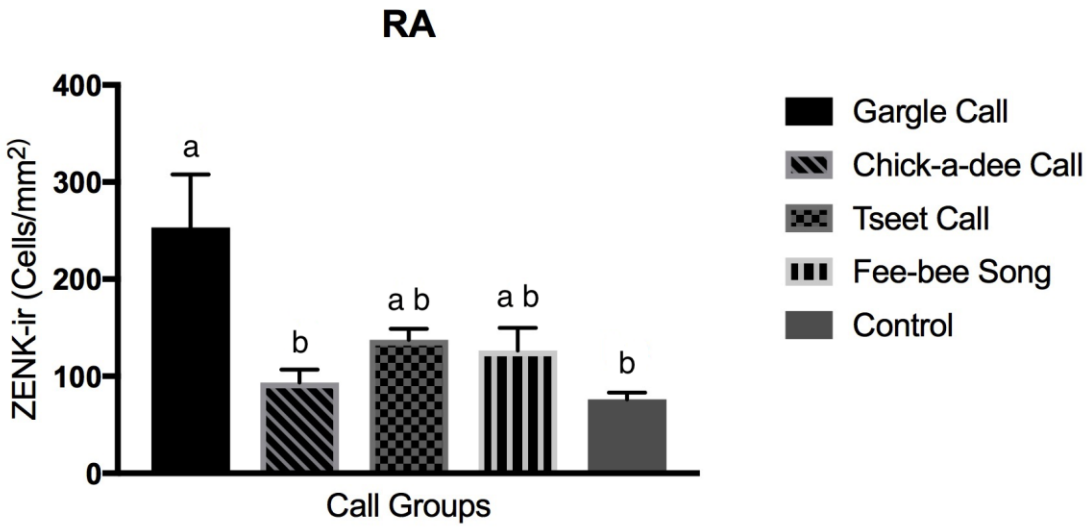

Figure 2- 7 Effect of vocalization type on the total number of ZENK-ir cells in RA of adult black-capped chickadees. The birds in the *gargle* call group had more ZENK-ir cells in RA than the *chick-a-dee* call, and the control groups. The letters represent statistical differences between the groups; letters that share the same lower case letter did not significantly differ from each other.

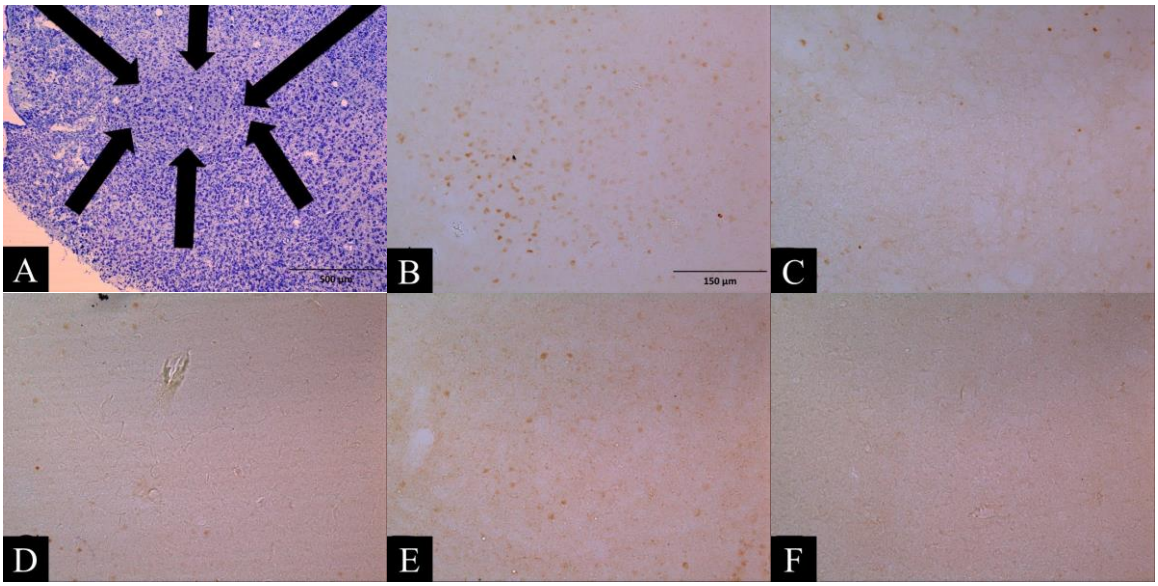

Figure 2- 8 Example ZENK immunoreactivity in the robust nucleus of the arcopallium (RA) of black-capped chickadees to each of the five vocalization conditions. A) Sagittal section of Nissl stained RA. B) ZENK immunoreactivity of black-capped chickadees producing *gargle* calls, C) *chick-a-dee* calls D) *tseet* calls and E) *fee-bee* songs. F) ZENK immunoreactivity of the silent black-capped chickadee control. Images B, C, D, E, F are all taken at the same magnification, and use the same scale. Caudal is to the left and anterior is toward the top of each image.

2.4 Discussion

This study was conducted to determine (a) if the song-control nuclei HVC and RA were involved in the production of the *fee-bee* song, the *gargle*, *chick-a-dee* and *tseet* calls, and (b) if they were involved, would there be any differences in ZENK-ir for the different vocalizations. The data do support the conclusions that HVC and RA are in fact involved in the production of calls, not just song. However the results suggest that there are differences in the amount of ZENK-ir in HVC and RA depending on which vocalization was produced. One interpretation of the results, HVC and RA ZENK-ir is a result of the number of vocalizations produced and not the type of vocalization. Then the most number of calls produced would result in the most ZENK-ir. However this is not the case, there was very low ZENK-ir for the *tseet* call, which was produced the most. The *gargle* call was the only vocalization to correlate with the amount of ZENK-ir in HVC and RA.

2.4.1 HVC

The *gargle* call was the only vocalization to show a significant correlation with the amount of ZENK-ir in HVC. This indicates that neurons within HVC are constantly firing during the production of the *gargle* call, and the more *gargle* calls are produced, the more neural activation is observed in HVC. Also when comparing the activation in HVC across the vocalization groups, the birds who were producing the *gargle* call showed the most activation, which was significantly more than the birds producing the *tseet* calls, *fee-bee* songs, and the silent control birds. However, the birds producing the *gargle* call did not differ in ZENK-ir in HVC from the birds producing the *chick-a-dee* call. These results are contrary to those obtained by Roach and colleagues (2016). In that

study, black-capped chickadees were exposed to four variations of the *fee-bee* song in a playback experiment. They also measured the amount of vocal production during these playbacks, and measured activity in HVC, but found that there was no correlation with the type or amount of vocalizations and ZENK-ir (Roach, Lockyer, Yousef, Mennill, & Phillmore, 2016). However, since these vocalizations were produced incidentally during playbacks of *fee-bee* stimuli, only a small number of vocalizations were produced. In my study, the number of vocalizations were much greater (i.e., *gargles* (min = 6, max = 167), *chick-a-dees* (min = 4, max = 65), *tseets* (min = 135, max = 490), *fee-bees* (min = 3, max = 54). This may have allowed me to pick up on differences that were impossible with such a small number of vocalizations in the study by Roach and colleagues (2016).

HVC is the first nucleus in the motor pathway for song production, it encodes for higher order song structure, and its neurons typically fire hundreds of milliseconds earlier than those in RA prior to the onset of song (Yu & Margoliash, 1996). Based on the pattern of activation observed, it seems likely that call complexity may play a role in HVC activation in the black-capped chickadee. The vocalizations of the black-capped chickadee can be arranged in terms of acoustic complexity (based on note characteristics, length, harmonic components etc.). Therefore, the hierarchical structure of chickadee vocalization complexity is as follows from most to least complex: the *gargle* call, the *chick-a-dee* call, the *fee-bee* song, and the *tseet* call. When examining the amount of neuronal activation within HVC for the different call types, we see the most activation for the most complex call, the *gargle*, and the least activation for the simplest call, the *tseet*.

2.4.2 RA

The *gargle* call was the only vocalization to show a significant correlation with the amount of ZENK-ir in RA, indicating that neurons within RA are constantly firing during the production of the *gargle* call, and the more *gargle* calls that were produced, the more activation was observed in RA. Also when comparing the activation in RA across the vocalization groups, the birds who were producing the *gargle* call showed the most activation, which was significantly more than the birds producing the *chick-a-dee* calls and the silent control birds. However, the birds producing the *gargle* call did not significantly differ in ZENK-ir in RA from the birds producing the *fee-bee* songs and *tseet* calls. Although the effect was not as pronounced across groups for neural activation in RA, the same trend is observed. The most activation was seen for birds that were producing the *gargle* call. This is unsurprising as RA is a structure that has been shown to be involved in call production in a bird model species, the zebra finch (Benichov et al., 2016; Ter Maat, Trost, Sagunsky, Seltmann, & Gahr, 2014; Vicario, Naqvi, & Raksin, 2001; Vicario, 2004). This activation may reflect the role of RA in the production of acoustically complex vocalizations. RA shows the most ZENK-ir for the *gargle* call, which is the most acoustically complex call that was measured in this study for the black-capped chickadee. The ZENK-ir also reflects the pattern of acoustic complexity, where the most is observed for the *gargle* call compared to the *tseet* call.

2.4.3 Conclusions

It is not surprising that both HVC and RA are involved in the production of calls in the black-capped chickadee, as this phenomenon has been previously observed in zebra and Bengalese finches (Ter Maat et al., 2014; Urbano, Aston, & Cooper, 2016).

The *gargle* call of the black-capped chickadee is acoustically complex, and is produced throughout the year (Ficken et al., 1978). And because HVC and RA are involved in the production of this call, it may explain why we do not see seasonal variation in the size of these song-control nuclei; these nuclei are being maintained year-round to support the production of calls. HVC and RA are part of the motor pathway in the song-control system and therefore it seems plausible that they would be involved in the production of a highly complex vocalization. In particular even suboscine species like the eastern phoebe (*Sayornis phoebe*) and the scale-backed antbird (*Willisornis poecilinotues*), have a rudimentary RA-like structure, which may have been an evolutionary predecessor to the complete song-control system observed in oscine species (Liu, Wada, Jarvis, & Nottebohm, 2013; De Lima et al., 2015). Although the *fee-bee* song in black-capped chickadees depends completely on learning, its production does not induce the most ZENK-ir, highlighting the fact that the song-control system may be related to acoustic complexity during production, and not the amount of learning required to learn the vocalization initially. Overall the song-control system may play a larger role in the production of more acoustically complex vocalizations, compared to simpler ones. Future studies should investigate exactly how these structures are involved in the production of these different calls, and specifically if the complex portions of these vocalizations are dependent on the functioning of these structures. If they are similarly involved in calls as they are in song, then HVC damage would abolish calling behaviour, and RA damage would seriously impact call structure (Nottebohmn et al., 1976).

2.5 References

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Chapter 3

3 HVC lesions have detrimental effects on the production of learned calls in black-capped chickadees

3.1 Introduction

Since the discovery of the song-control system in the 1970s, the neural basis of song learning and production has been the primary focus of neurobiology research in songbirds (Catchpole & Slater, 2008; Kroodsma & Miller, 1996, Marler, 2004). These studies have focused on understanding how this set of discrete brain nuclei are involved in the learning and production of birdsong and, to a lesser extent, song perception. A particular nucleus, HVC (not an acronym, though sometimes referred to as the high vocal center), was found to be crucial for song production: when HVC was lesioned bilaterally in canaries, they were no longer able to sing, but would still move their beaks as if they were attempting to sing (Nottebohm, Stokes, & Leonard, 1976). In addition, electric stimulation of HVC during singing would stop the song, and birds would restart it from the beginning (Vu, Mazurek, & Kuo, 1994). Based on these and numerous other studies it is well-known that HVC is crucial for the production of song (for review see Nottebohm, 2005). However, although this structure has a well-established involvement in birdsong production, its involvement in bird call production is relatively unclear.

Unlike birdsong, which is learned early in life, we know that bird calls can be innate, learned, or partially learned (for review see Marler & Slabbekoorn, 2004; Vicario, Raksin, Naqvi, Thande, & Simpson, 2002). Imitative vocal learning, whether of songs or calls, is observed throughout the animal kingdom: in songbirds, elephants, parrots, bats, whales, primates and seals (Doupe & Kuhl, 1999). Calls serve a much more varied

purpose than birdsong (used for courtship and territory defense), and are used in a variety of social contexts such as maintaining contact with the members of one's group, displaying aggressive behaviours, or announcing the presence of food or a predator. Calls are therefore crucial to an animal's survival (Tyack, 2008). Black-capped chickadees produce a variety of calls, such as the *chick-a-dee* and *gargle* calls, in addition to their song, the *fee-bee* (for complete repertoire see Ficken, Ficken, & Witkin, 1978).

The *chick-a-dee* call is used both as a contact call, to maintain contact with members of their group, as well as a mild alarm call when a predator is nearby (Ficken et al., 1978). There is some evidence that indicates that the *chick-a-dee* call is partially learned (Baker, Baker, & Gammon, 2003; Clemmons & Howitz, 1990; Hughes, Nowicki, & Lohr, 1998). Black-capped chickadees that are raised in both social and acoustic isolation have abnormal *chick-a-dee* calls, they produce fewer B and C notes, and when they produce these notes they are acoustically different from wild type chickadee B and C notes (Hughes et al., 1998). Therefore, the acquisition of a species-typical *chick-a-dee* call requires auditory input from conspecific birds. It therefore seems likely that if chickadees require auditory input and learning to produce species typical B and C notes, that the song-control system is involved in this process and specifically that HVC is involved. This hypothesis remains untested.

The *gargle* call is used as an aggressive vocalization, usually to advertise an imminent attack on another bird (Ficken et al., 1978). Chickadees found in different geographic regions produce different types of *gargle* calls, and each individual chickadee has a repertoire of up to 10 distinct *gargles*, comprised of up to 10 syllables, therefore producing on average approximately 60 distinct *gargle* syllables (Baker, Baker, &

Gammon, 2003; Baker & Gammon, 2008; Baker, Howard, & Sweet, 2000; Ficken, Ficken, & Apel, 1985; Ficken & Weise, 1984; Ficken et al., 1987). Although there is no direct evidence that the *gargle* call is learned, the differences in the structure of *gargle* calls across different geographic locations would suggest that some learning likely occurs in order to produce the geographically distinct *gargle* call dialects. This suggests that, as for the *chick-a-dee* call, the song-control system may be involved in the development and production of the *gargle* call.

In order to understand the role of HVC and other song nuclei in the perception and production of birdsong, a variety of lesion studies have been conducted (Burt, Lent, Beecher, & Brenowitz, 1999; Genter, Hulse, Bentley, & Ball, 1999; Halle, Gahr, & Kreutzer, 2003; Nottebohm, Stokes, & Leonard, 1976; Sohrabji, Nordeen, & Nordeen, 1990; for review see Konishi, 1985). These types of studies allow us to examine the behavioural impact of inactivating a particular neural structure. For example, canaries with right-hemisphere and left-hemisphere HVC lesions show detrimental effects on song production; however, these effects vary depending on the hemisphere lesioned (Halle, Gahr, & Kreutzer, 2003). Right hemisphere lesions reduced the highest frequency and the widest frequency band in songs whereas left hemisphere lesions increased the lowest frequency of songs. The size of the left hemisphere lesions also correlated with a reduction in the number of simple syllables produced in the song, as well as a decrease in the total number of songs in the repertoire. Therefore, HVC lesions have specific effects on the acoustic parameters of song, in addition to the overall abolishment of song with complete bilateral lesions (Halle, Gahr, & Kreutzer, 2003).

Immediate-early genes are a tool that can allow us to investigate whether or not a

particular region of the brain is active during particular behaviours (Jarvis, Ribeiro, da Silva, Ventura, Vielliard, & Mello, 2000). They have been used to show that hummingbirds have song-control nuclei, and that these are active when they are singing. In Chapter 2, I used the immediate-early gene ZENK to determine the amount of activation in HVC and RA, if any, during call production. However, one of the limitations of examining ZENK immunoreactivity (ZENK-ir), as in Chapter 2, to determine if a brain region is active during vocal production, is that the activation could be due to the auditory perception of the vocalization or the production of the vocalizations. By conducting a lesion experiment, we can dissociate between these two possibilities. In Chapter 2, I found the most ZENK-ir following production of *gargle* calls, closely followed by that following the production of *chick-a-dee* calls. These results suggest that HVC is likely involved in the production of the *gargle* and *chick-a-dee* calls, and bilaterally lesioning HVC would dissociate whether this result was due to the perception of the vocalization or the production.

The objective of this study was to examine the effect of bilateral excitotoxic HVC lesions on the production of the *gargle* and *chick-a-dee* calls. Excitotoxic lesions are superior to electrolytic lesions because they preserve the fibers of passage across nuclei; destruction of fibers of passage across the structures can confound the interpretation of HVC lesions. To meet my objectives in this study I captured black-capped chickadees and put them in social and acoustic isolation from one another before exposing them to various stimuli in order to elicit *gargle* and *chick-a-dee* calls to provide a baseline measure of these vocalizations before the HVC lesion surgery was conducted. The birds were then subjected to an HVC lesion surgery where they were injected bilaterally into

HVC with ibotenic acid. After recovery, birds were again exposed to stimuli to elicit *gargle* and *chick-a-dee* calls post-lesion. The birds were then euthanized and the brains were examined to determine the location of the lesions. The *gargle* and *chick-a-dee* calls were compared, pre-lesion to post-lesion, using bioacoustic measures.

I predicted that chickadees with bilateral HVC lesions would have impaired production of *gargle* and *chick-a-dee* calls post-lesion. Specifically, for the *chick-a-dee* call I predicted that B and C notes would be strongly affected by HVC lesion but that A and D notes would remain relatively unchanged. This prediction follows the observation that the A and D notes are relatively unaffected when chickadees are raised in acoustic and social isolation, and therefore are most likely innate (Hughes et al., 1998). I predicted that *gargle* calls would be more inconsistent post-lesion, specifically that there would be fewer notes in the *gargle* calls. For both call types I predicted that there would be a reduction in the number of notes post-lesion and that there would be a decrease in the highest frequencies of the notes, and an increase in the lowest frequency, based on similar results in single hemisphere lesions on canary song (Halle et al., 2003). Finally, I predicted that birds who had lesions that missed HVC in both hemispheres would show little to no differences in the structures of their *gargle* and *chick-a-dee* calls.

3.2 Methods

3.2.1 Subjects and housing

During the winter season from September 2014 to September 2016, I captured 17 adult black-capped chickadees (*Poecile atricapillus*) at the University of Western Ontario Campus, London, Ontario (43˚01’ N, 81˚27’ W). Only male birds were used; they were

identified as male by using body mass and wing chord measurements, and sex was later confirmed by examining the gonads post-mortem. In order to acclimatize the birds to captivity and to assess the birds' physical condition, they were quarantined and group housed (range: 3-4 birds per cage) in rooftop aviaries for two weeks. Birds had ad libitum access to food (Mazuri small-bird maintenance diet mixed with black-oil sunflower seeds) and water; their diet was also supplemented with mealworms (2 worms per individual per day).

Following the quarantine, birds were put into social and acoustic isolation in a wire cage lined with newspaper placed inside a modified audiometric testing booth (width 91cm X height 172cm X depth 71cm, Industrial Acoustics Company, Inc., Bronx, NY). The birds had ad libitum access to food and water in the chamber. The photoperiod inside the isolation chamber was set to match the outdoor ambient daylight cycle. The birds remained in isolation for a period of at least 48 hours before recording their vocalizations to establish a baseline repertoire.

The final sample size for this study was 6 birds, which may seem like a small number, but unlike most animal studies, lesion studies tend to have a smaller number of total subjects, due to the invasive nature of the experiments. It is typical to have between 5 and 10 subjects for a lesion study (Bottjer, Miesner, & Arnold, 1984; Burt et al., 1999; Genter et al., 1999; K. S. Lynch et al., 2012; McCasland & Konishi, 1981; Nottebohm et al., 1976; Sohrabji et al., 1990). One of the birds died due to issues with the isoflurane anesthetic (first bird to undergo surgery received 2.5% isoflurane and died during surgery, the anesthetic was adjusted in subsequent surgeries). Another died due to a surgical complication (hitting a major blood vessel in the brain leading to massive

intracranial hemorrhage). The other 9 birds in the study were used to pilot the lesion surgery, and specifically to determine the technique and coordinates that would work. I used wild-caught black-capped chickadees and unlike inbred lab species, the structures within the brain vary in location considerably, just as they do across humans. These birds were used to determine the coordinates that worked most consistently and the technique of the needle insertion and retraction before infusing the ibotenic acid.

3.2.2 Behavioural recordings

Following the isolation period, all food and water cups were removed and the birds were presented with two different stimuli on the first day and two on the second day, in order to elicit the *gargle* call and *chick-a-dee* call to get a baseline of these vocalizations for comparison post-lesion. The sessions were recorded using a Marantz PMD 671 recorder attached to a Sennheiser ME62 microphone to record vocalizations and a JVC handheld video camera (GZ-MS120) to monitor behaviour. The birds were first presented with an unfamiliar chickadee with a cup of sunflower seeds placed in the center of the cage for 15-min. Both birds were placed inside the same wire cage, and were identified in video recordings based on their coloured leg bands. This scenario was devised in order to incite an aggressive encounter between the two individuals, in which *gargle* calls are often produced (Smith, 1991). The number and variety of calls was quantified, and later confirmed when listening and viewing recordings of the session. When presented with an unfamiliar chickadee, all birds produced the *gargle* call, and produced a minimum of 12 calls during the 15-min session. The stimulus was then

removed, food and water dishes were returned and the bird was left in isolation for at least 15-min.

Following the isolation period, the food and water dishes were removed and the chickadee was presented with a taxidermy saw-whet owl (*Aegolius acadicus*) for a period of 15 min, in order to elicit the *chick-a-dee* call. The *chick-a-dee* call is a mild alarm call, and is typically given when presented with a predator, sometimes accompanied by a *high-zee* call (Smith, 1991) The session was video and audio recorded as above, and the number and variety of calls was quantified, and later confirmed when listening and viewing recordings of the session. When presented with the taxidermy saw-whet owl, all birds produced the *chick-a-dee* call, and produced a minimum of 12 calls during the 15-min session. The stimulus was then removed, food and water dishes were returned and the bird was left in isolation overnight.

The next morning following overnight isolation, the food and water dishes were removed and the chickadee was presented with one mirror on either side of its cage (12 cm x 12 cm) for 15-min. The session was video and audio recorded, and the number and variety of calls was quantified, and later confirmed when listening and viewing the recordings or the session. When presented with the mirrors, birds produced the *chick-a-dee* call, the *gargle* call or the *tseet* call, or a combination of the aforementioned. These stimuli were used in order to mimic the presence of multiple birds inside the cage (either mimicking an aggressive/dominant interaction, or a flock interaction) and to obtain additional samples of each vocalization as *chick-a-dee* and *gargle* calls can vary depending on the context (Smith, 1991). At the end of 15-min the stimuli were removed, food and water dishes were returned and the bird was left in isolation until 12:00.

Following isolation the bird inside the wire cage was moved upstairs into the outdoor aviary and placed on the floor of an aviary containing multiple chickadees, and was allowed to acclimatize for one hour. This was done in order to mimic a true social situation where the birds were surrounded by many chickadees that they could both see and hear around them, mimicking situations in which they are in flocks, and are extremely social (Smith, 1991). Subsequently, the bird was audio and video recorded in the outdoor aviary for 25 min. Following the recording session the bird was returned to isolation until the following morning where they were subjected to an HVC lesion surgery.

3.2.3 HVC lesion surgery

I injected birds intramuscularly with analgesic (0.01 mL of 0.625 mg/mL meloxicam). Birds were then anesthetized with 2.5% isoflurane at a flow rate of 2 L of oxygen per minute, and I securely placed their heads in a stereotaxic mount, where a drill and 1 μL Hamilton syringe were mounted. I removed the feathers along the central part of the skull by using 70% ethanol, I disinfected the skin with a microbicide (Betadine ®), and again applied 70% ethanol. I applied a small amount of topical local anesthetic (mix of lidocaine and prilocaine, EMLA® cream) to the skin. I made an incision of 0.75 cm in length along the midline and exposed the skull; I then positioned the drill bit at the tip of the central sinus that was used as the fronto-caudal marker for the stereotaxic coordinates.

I moved the drill 2.1 mm lateral from the central sinus to the left hemisphere, and drilled a hole into the skull exposing the brain (see Figure 3-1). I pierced through the meninges

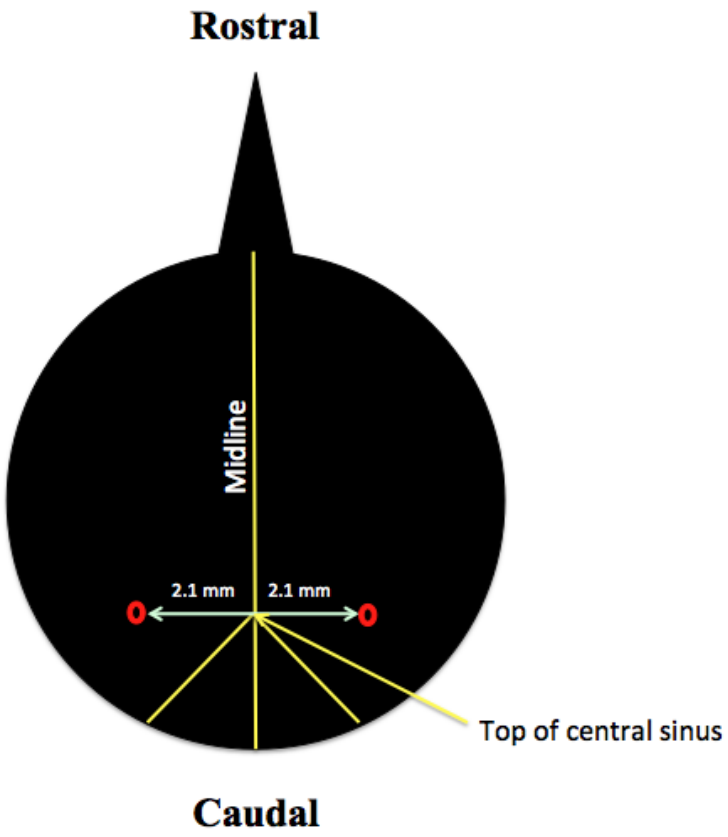


Figure 3- 1 Diagram of the black-capped chickadee head during surgery. The midline, and central sinus that were used as markers for the stereotaxic measurements for the drill placement are depicted. The red circles show the locations where the skull was perforated with the drill and the Hamilton syringe was inserted. These measures were the same for all birds.

using a 26-gauge needle tip. I repeated the same procedure for the right hemisphere. These coordinates were determined by trial and error with different individuals. I aligned the Hamilton syringe with the hole in the skull and lowered the syringe into the brain 2mm in depth, and then retracted to 1mm in depth. Over a period of 3-min I infused 0.2 µL of a glutamatergic neurotoxin (1% ibotenic acid in phosphate buffered saline; Sigma;

St. Louis, Mo.). I retracted the Hamilton syringe and repeated the procedure in the right hemisphere. I then closed the skin using a tissue adhesive (3M Vetbond™), and returned the birds to their home cages inside individual isolation chambers, where they were allowed to recover for 3 days, and received 0.01 mL of 0.625 mg/mL meloxicam each of the 3 days.

3.2.4 Post-surgery behavioural recordings

After 3 days, the birds were presented with the same stimuli (i.e., unfamiliar chickadee, taxidermy saw-whet owl, mirrors, and outdoor aviary) and the number and variety of calls was quantified, and later confirmed when listening and viewing recordings of the session. However if a bird failed to produce the *gargle* call during the unfamiliar chickadee stimulus or the *chick-a-dee* calls during the taxidermy saw-whet owl stimulus session, these stimuli were repeated on a subsequent day for a maximum of three sessions. Only one session of the mirror stimuli, and one of the outdoor aviary stimulus was recorded post-surgery for each individual bird. Following the last recording session, I euthanized the birds using an overdose of isoflurane. The fresh brain was then quickly removed from the skull and immediately frozen on crushed dried ice and then stored at -80 ˚C. Prior to histological analyses, each brain was cut in half along the sagittal plane and both the left and right hemisphere were used for subsequent analyses.

3.2.5 Bioacoustic analysis of pre- and post-surgical calls

Using RavenPro 1.4 (Bioacoustics Research Program, 2011), and plotting the spectrograms of each recording session, I verified the number of songs and calls produced in each recording for each bird tested, as well as identifying the type of

vocalization produced. Signal™ 5 (Digital Signal Analysis System, 2015) was used to measure the acoustic structure of the *chick-a-dee*, and *gargle calls*.

3.2.5.1 *Chick-a-dee* calls

Chick-a-dee calls were categorized into one of three possible categories; complete *chick-a-dee* calls (containing at least one A, B or C note, as well as at least one D note), ABC only calls (which did not contain any D notes), or D only calls (which only contained D notes). For the purposes of this study only complete *chick-a-dee* calls were measured. A random (using https://www.random.org/lists/) sample of 10 complete *chick-a-dee* calls was obtained from the pre-lesion recordings. If the complete *chick-a-dee* call was produced in more than one recording session, then the calls were obtained from each recording, making sure that equal numbers of complete *chick-a-dee* calls were obtained from the sessions. The same procedure was used for sampling the *chick-a-dee* calls in the post-lesion recordings. In some cases there weren't enough complete *chick-a-dee* calls to make up the sample of 10 calls, in which case all complete *chick-a-dee* calls produced were used. Birds GrPe.O, WhWh.OO, RG.lB, and BGr.Y had the total number of *chick-a-dee* calls for analyses (10 pre-lesion, 10 post-lesion). However birds lB.Bl and Br.O had a samples of 14 complete *chick-a-dee* calls (10 pre-lesion, 4 post-lesion each).

The bioacoustic features I measured were based on the methods described in Charrier, Bloomfield, & Sturdy (2004) and Nowicki & Nelson (1990). The measurements included: start frequency (SF in Hz), end frequency (EF in HZ), peak frequency (PF in Hz), and note peak frequency (NPF in Hz, the highest frequency in the highest harmonic when additional harmonics occur). These characteristics were measured on a digital spectrogram (window size = 1024 points, frequency precision = 43 Hz) (see Figure 3-2).

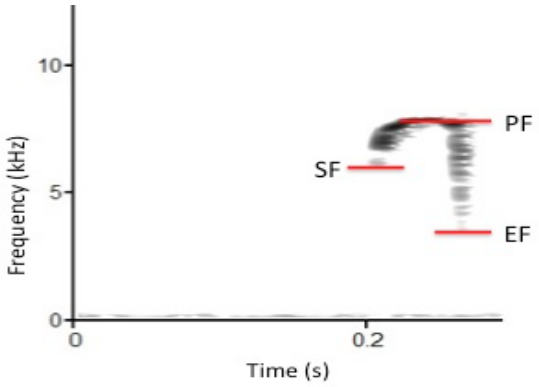


Figure 3- 2 Spectrogram showing the variables measured on A, B, and C notes, depicted at high frequency in order to assess start frequency (SF), peak frequency (PF) and end frequency (EF). The x-axis depicts time, and the y-axis depicts the frequency in Hz.

Measurements on A and B notes were made on the primary (highest amplitude) harmonic, whereas the measures for SF, PF and EF were made on the first visible harmonic for C notes. The maximal frequency was also measured (F_{max} in Hz) using a power spectrum (see Figure 3-3). Duration measures were also taken; these included total

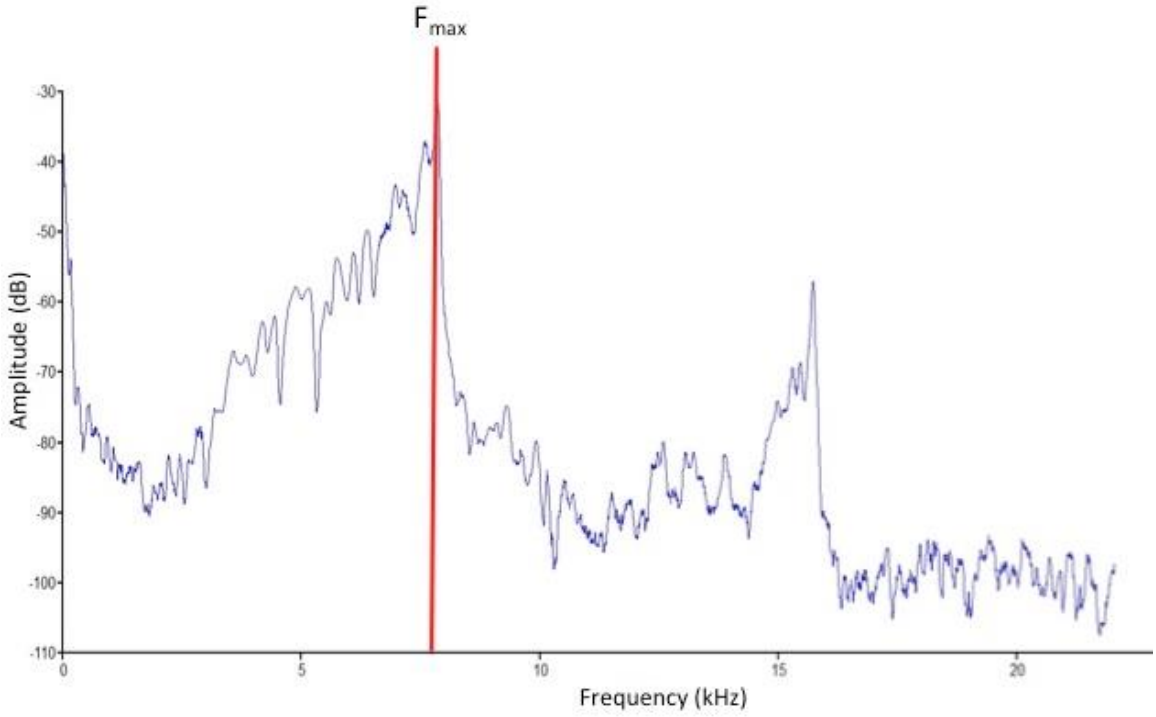


Figure 3- 3 Power spectrum depicting a non-D note, used to measure the highest frequency in the note (F_{max}). Frequency is depicted on the x-axis in Hz, and amplitude in dB is depicted on the y-axis.

call duration (TCD in ms), total note duration (TD in ms), as well as ascending duration (AD in ms), and descending duration (DD in ms) (see Figure 3-4). These were measured

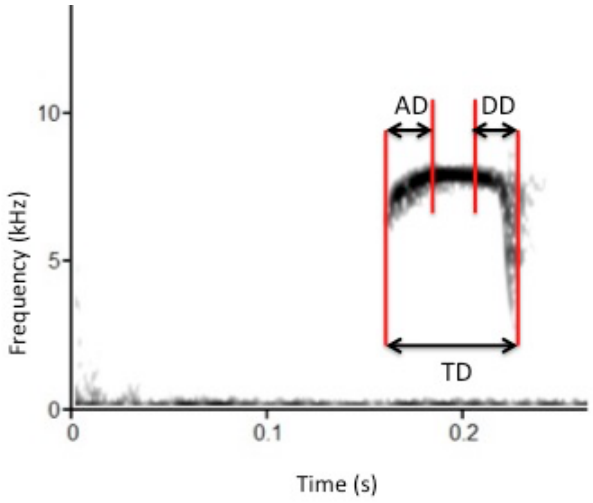


Figure 3- 4 Spectrogram of non-D notes resolved at high time to assess the variables of total note duration (TD), ascending duration (AD) and descending duration (DD). The x-axis depicts time, and the y-axis depicts the frequency in Hz.

on a digital spectrogram (window size = 256 points, temporal precision = 5.8 ms). For the D notes, I measured four different acoustic features, including total duration (TD) (see Figure 3-5), frequency of the first visible harmonic (f_0 in Hz), maximal frequency (F_{max} in

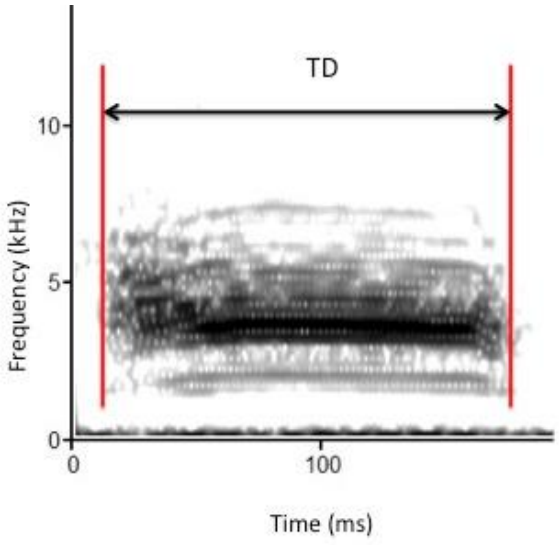


Figure 3- 5 Spectrogram of D notes resolved at high time to assess TD. The x-axis depicts time, and the y-axis depicts the frequency in Hz.

Hz) and NPF (see Figure 3-6). The frequency measures were obtained using a power spectrum with a fast Fourier transform window size of 16 384 points, and a frequency precision of 2.7 Hz (smoothing width = 88.2 Hz).

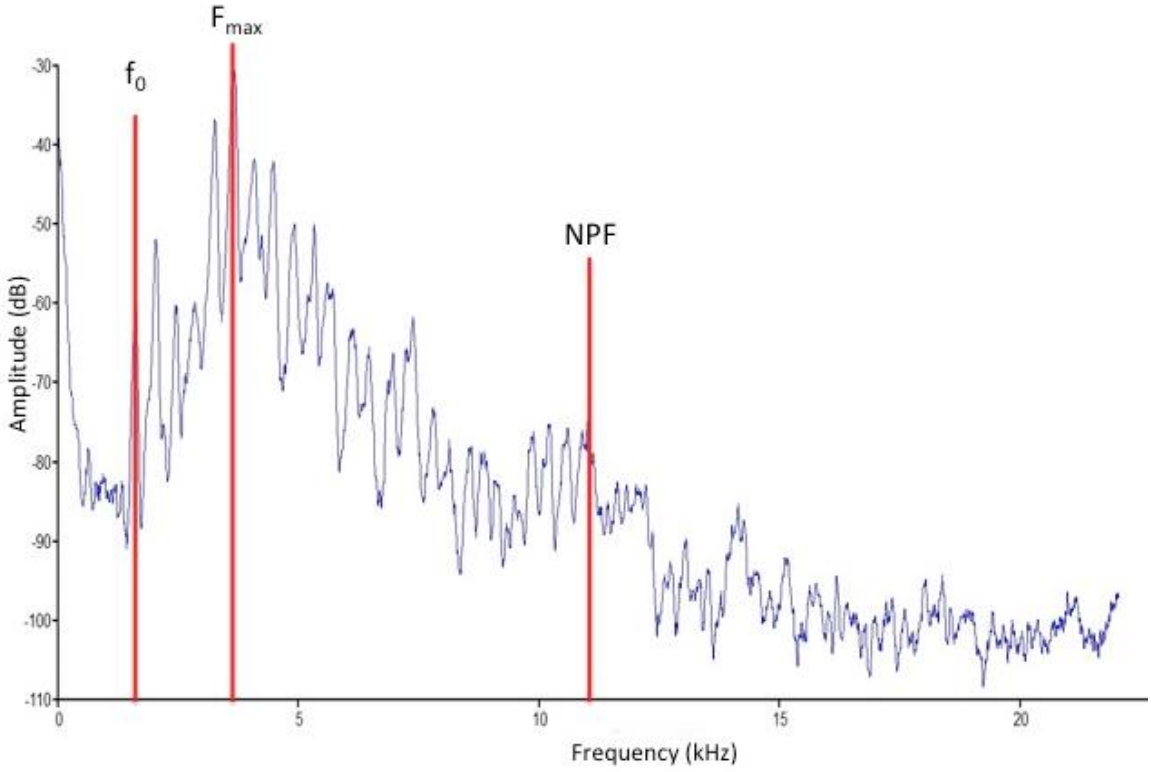


Figure 3- 6 Power spectrum depicting a D note, used to measure the maximal frequency in the note (F_{max}), the first visible harmonic (f_0), and the note peak frequency (NPF). Frequency is depicted on the x-axis in Hz, and amplitude in dB is depicted on the y-axis.

3.2.5.2 *Gargle* calls

Gargle calls were categorized for each individual bird because *gargle* calls tend to be individually unique, although can share some components across individuals. *Gargles* were identified acoustically and by using the spectrograms produced by Signal™ 5 software (Digital Signal Analysis System, 2015). Pre-lesion the *gargle* calls were easily

identifiable and were classified into their respective types, however post-lesion the *gargle* calls varied greatly, and were matched up with their pre-lesion types based on syntactic classifications, however a great number of them were no longer identifiable post-lesion. A pseudo-random (using https://www.random.org/lists/) sample of 10 *gargle* calls was obtained from the pre-lesion recordings, if *gargle* calls were produced in more than one recording session, then the calls were obtained from each recording, making sure equal numbers of the *gargle* calls were obtained from the individual recordings. Post-lesion the *gargle* calls that were identifiable were matched for type, if possible, with the pre-lesion *gargles*, and were then sampled in the same manner to try and get a sample of 10 post-lesion *gargle* calls (see Table 3-1 for specific sampling numbers).

Table 3- 1 Table showing the number of *gargle* calls sampled for each type and for each individual bird. Birds Br.O and BGr.Y are control birds, whereas GrPe.O, WhWh.OO, lB.Bl and RG.lB are bilaterally HVC lesioned birds.

	***Gargle* call type**	**Number of calls sampled pre-lesion**	**Number of calls sampled post-lesion**
Br.O	97	10	5
	98	10	10
BGr.Y	88	10	7
GrPe.O	1	10	6
WhWh.OO	73	10	10
	74	10	10
	75	10	10
	76	10	10
lB.Bl	11	10	1
	12	8	3
	13	3	1
	17	9	2
RG.lB	2	10	7
	3	10	10
	4	10	7
	5	10	4

Since there is no standard method of measuring the bioacoustic features of the *gargle* call, I based my measurements on the works of Charrier, Bloomfield, & Sturdy (2004) and Nowicki & Nelson (1990) on *chick-a-dee* calls and modified it to measure the *gargle* calls. The measurements included: start frequency (SF in Hz), end frequency (EF in HZ), peak frequency (PF in Hz), top frequency (TF in Hz), middle frequency (MF in Hz) and bottom frequency (BF in Hz) and note peak frequency (NPF in Hz, the highest frequency in the highest harmonic when additional harmonics occur) (see Figure 3-7).

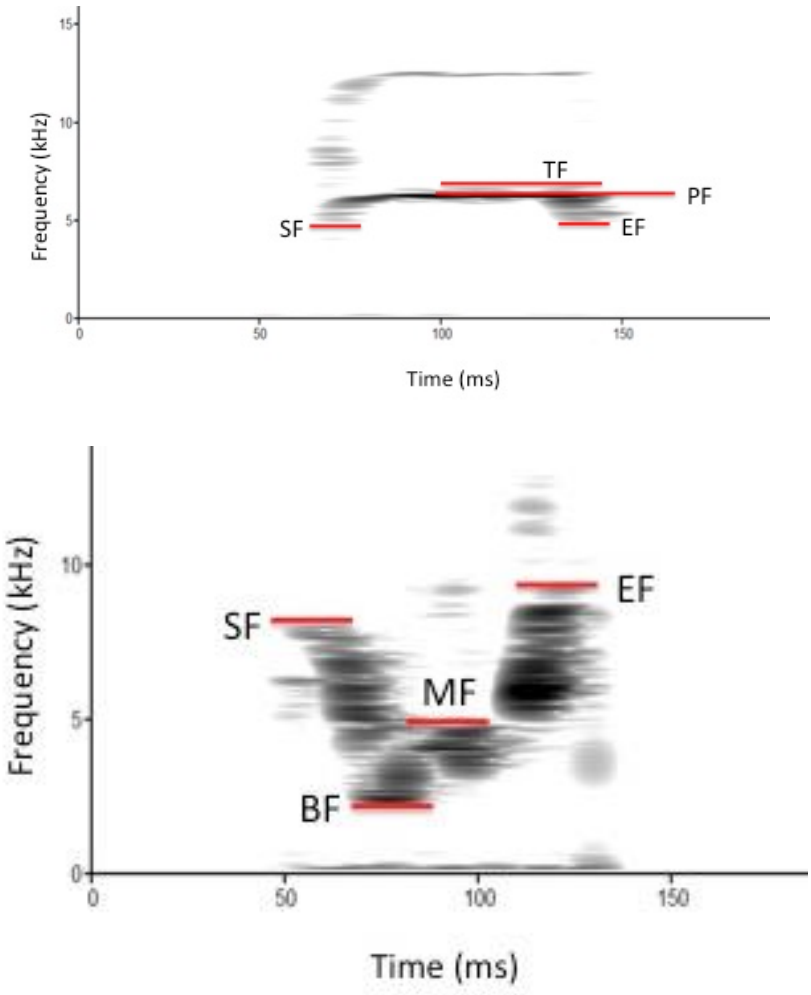


Figure 3- 7 Spectrograms depicting a non-harmonic note of *gargle* calls, depicted at high frequency in order to assess start frequency (SF), peak frequency (PF), top

frequency (TF), bottom frequency (BF) and mid- frequency (MF) and end frequency (EF). The x-axis depicts time, and the y-axis depicts the frequency in Hz.

Not all measures were possible to obtain in the different calls, in that case a subset of the measures were taken. These acoustic features were measured on a digital spectrogram (window size = 1024 points, frequency precision = 43 Hz). The maximal frequency was also measured (F_{max} in Hz) using a power spectrum (see Figure 3-8). Duration measures

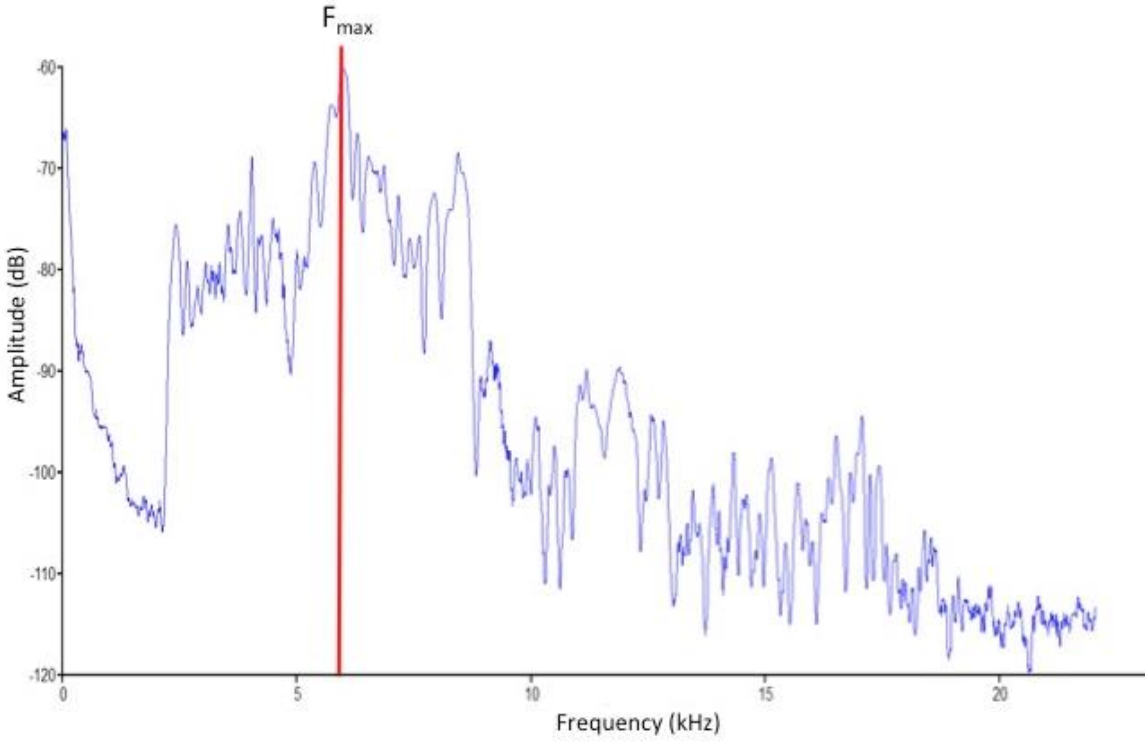


Figure 3- 8 Power spectrum depicting a non-harmonic note of *gargle* calls, used to measure the maximal frequency in the note (F_{max}). Frequency is depicted on the x-axis in Hz, and amplitude in dB is depicted on the y-axis.

were also taken; these included total call duration (TCD in ms), total note duration (TD), as well as ascending duration, (AD in ms), and descending duration, where applicable (DD in ms) (see Figure 3-9). These were measured on a digital spectrogram (window size

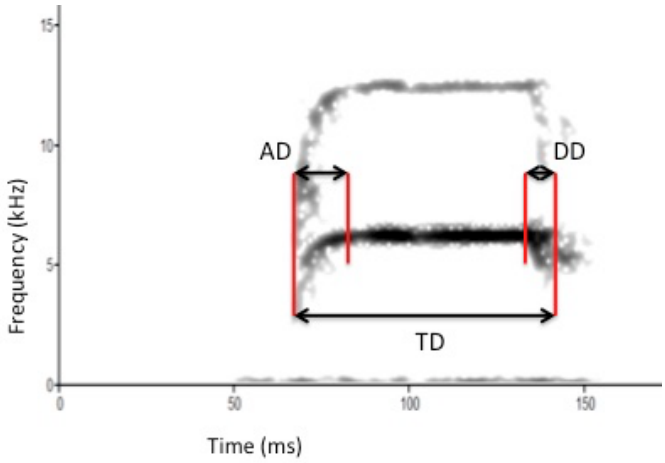


Figure 3- 9 Spectrogram of non-harmonic notes of *gargle* calls resolved at time to assess the variables of total note duration (TD), ascending duration (AD) and descending duration (DD). The x-axis depicts time, and the y-axis depicts the frequency in Hz.

= 256 points, temporal precision = 5.8 ms). For the harmonic notes, I measured four different acoustic features, including total duration (TD), frequency of the first visible harmonic (f_0 in Hz), maximal frequency (F_{max} in Hz) and NPF (see Figure 3-10). The frequency measures were obtained using a power spectrum with a fast Fourier transform window size of 16 384 points, and a frequency precision of 2.7 Hz (smoothing width = 88.2 Hz).

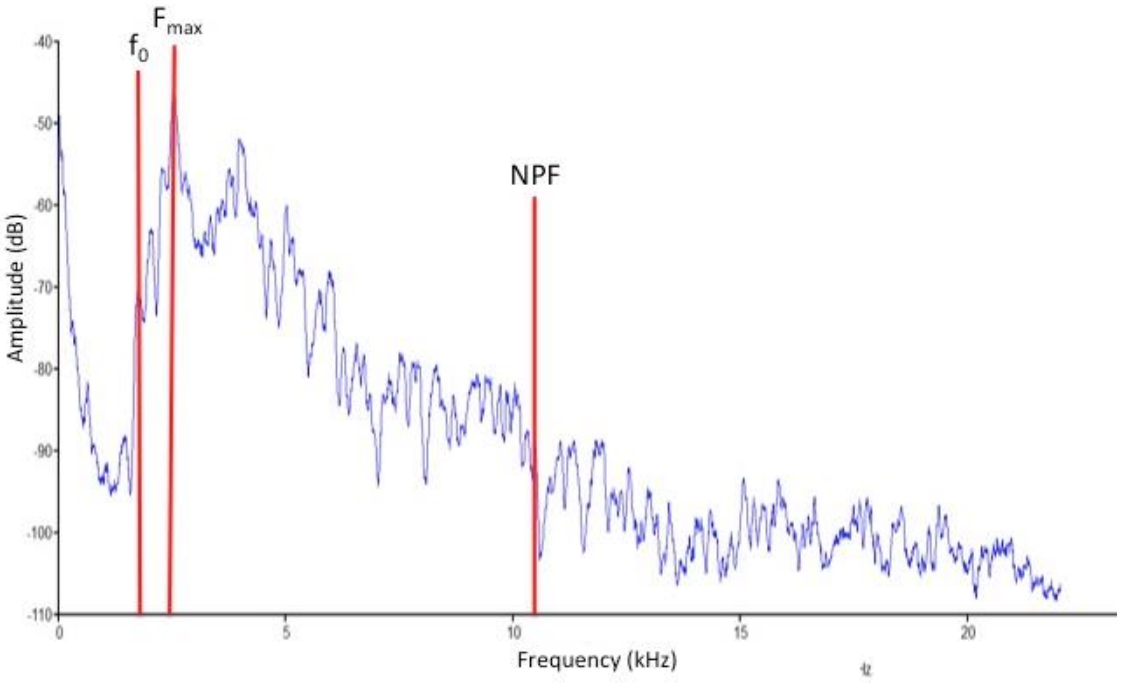


Figure 3- 10 Power spectrum depicting a harmonic note of *gargle* calls, used to measure the maximal frequency in the note (F_{max}), the first visible harmonic (f_0), and the note peak frequency (NPF). Frequency is depicted on the x-axis in Hz, and amplitude in dB is depicted on the y-axis.

3.2.6 Nissl histology and quantification

Using a cryostat I sectioned brains along the sagittal plane in 30 µm sections. I started thaw-mounting every other section once the cerebellum was visible onto electrostatically treated microscope slides (VWR VistaVision™ Histobond ®). The slide was dried on a slide warmer for 5-min before being submerged in 4% paraformaldehyde for 5-min, and left to air-dry overnight before processing them the following day.

Once dry, the slides were stained using thionin, followed by serial dehydrations with increasing concentrations of ethanol, and cleared of lipids with an organic solvent (NeoClear, cat no. 65038-71; EMD Chemicals, Mississauga, Ontario, Canada). Finally,

the slides were covered with coverslips using a mounting medium (Permount, cat no. SP15; Fisher Scientific) and allowed to dry in the fume hood ~ 24 h. I determined the location of HVC and the lesions by using a Leica DM 5500B microscope coupled to a Leica 420C camera. For each chickadee, a minimum of 21 images (n = 6, M = 30.12, SD = 7.19) were captured using both the 1.25x and 5x objective lens, of all sections containing a lesion, as well as images of intact HVC if the lesion had missed. The sections were selected such that the middle of the imaged section contained the largest cross-section of HVC with the lesion clearly visible. The lesions were therefore classified as either a 'hit' or a 'miss'. A hit was recorded if the lesion damaged at least part of the HVC in both hemispheres (see Figure 3-11), whereas a miss was recorded if no part of HVC was damaged in either hemisphere. The lesions were then classified into 2 categories; hit/hit (n = 4), and miss/miss (n =2). Birds that had a hit in one hemisphere and a miss in the other were not analyzed for this thesis.

A lesion was considered successful if it had affected HVC in both the left and right hemisphere, this is because neurochemical lesion studies have shown that the location of the lesion within HVC doesn't affect the effectiveness at producing behavioural effects, rather it is the integrity of HVC itself that matters (Del Negro, Gahr, Leboucher, & Kreutzer, 1998).

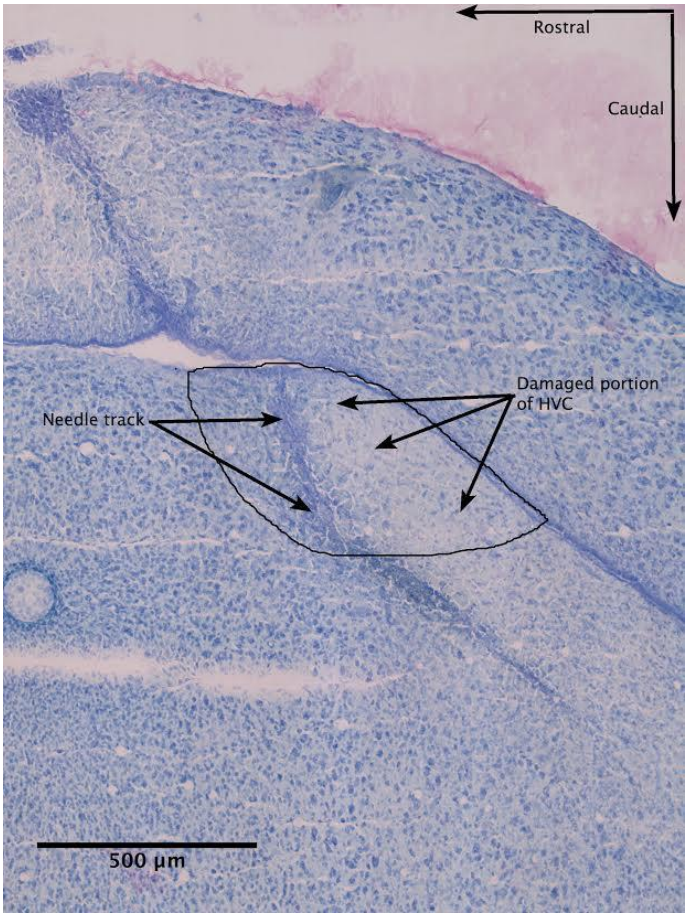


Figure 3- 11 Sample image of Nissl stained lesioned HVC. Depicted is the trajectory of the needle, the lighter portion of HVC depicting the damage caused by the ibotenic acid.

3.2.7 Data and statistical analyses

Statistical analyses were carried out using IBM SPSS Statistics 24.0. Because each bird produced unique *gargle* calls it was not possible to compare the calls between groups. Thus I compared each unique *gargle* call to itself before and after the lesion using t-tests for each individual bird. Results were considered significant at $\alpha \leq 0.05$ level. Data are presented as t-values and percent changes (PC), which were calculated by using the following formula:

$$\frac{\{\text{Mean value of measure pre-lesion} - \text{Mean value of measure post-lesion}\}}{\text{Mean value of measure pre-lesion}} \times 100$$

3.3 Results

Due to the large amount of variation of the *gargle* calls across individuals, the results for both the *gargle* and *chick-a-dee* calls are presented on a case-by-case basis for each individual bird in the experiment. The *gargle* call results are presented first followed by the *chick-a-dee* call results.

3.3.1 *Gargle* calls

3.3.1.1 Bilateral lesioned birds

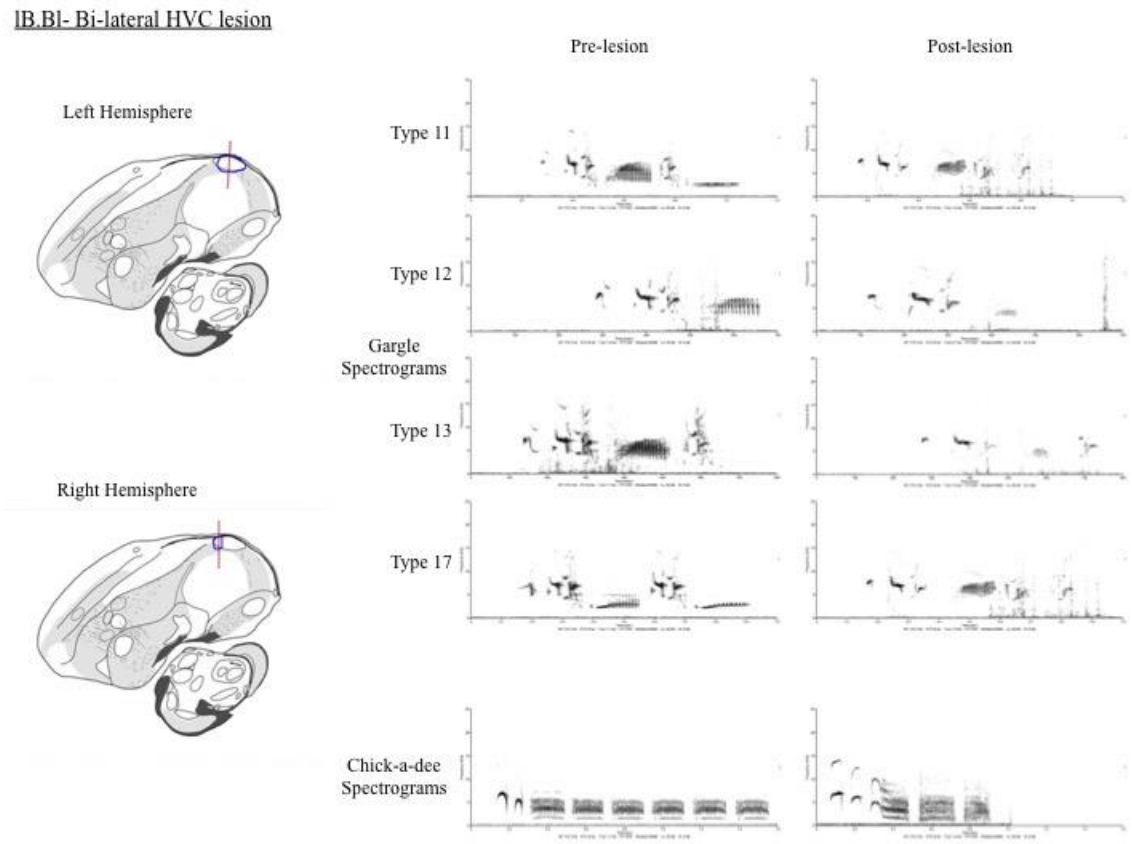
In general, HVC lesions made the *gargle* calls shorter, as much as 43% shorter. There were also changes in the harmonic structures post-lesion, they were more variable, and typically spanned a greater frequency range. The acoustically complex notes (see Figure 3-13, note 3), usually lost some of their acoustic complexity and became much simpler, and typically the end frequency increased and the top frequency of these note types decreased. Although pronounced effects were observed for the *gargle* and *chick-a-dee* calls post-lesion, there were also a number of unidentifiable call portions that were produced post-lesion that I was unable to identify or attribute to a particular call type (see panel B in Figures 3-12, 3-17, 3-22). These types of vocalizations were not present in the pre-lesion recordings of any of the birds and could not be measured for acoustic structure. Presumably these calls represent severely impaired attempts by the birds to produce normal calls. These attempted calls include some note observed in pre-lesion *gargle* calls, however the sequence did not match any known call that chickadee made when intact. These attempted calls also varied greatly, where for birds lB.Bl and WhWh.OO there were many more types but only a subset is presented (see panel B in Figures 3-11 and 3-16). In addition to these highly aberrant calls, calls that were identifiable by type were

acoustically different from pre-lesion calls. Examples of these changes are highlighted below, and complete descriptions of these changes are provided in Appendix A.

3.3.1.1.1 Bird lB.Bl

The lesion locations and example spectrograms of pre- and post-lesion calls are presented in Figure 3-12. This bird had lesions that damaged HVC in both hemispheres. Prior to lesions it produced 4 *gargle* call types, and post-lesion it produced 4 of those as well as a larger number unidentifiable calls (Figure 3-12, panel B). *Gargle* calls that were identified post-lesion generally had less complex acoustic structure with reduced harmonic structure in several notes (see Figure 3-12, panel A). Detailed examples are provided below and complete statistical comparisons of acoustic measures are provided in Appendix A.

A)


lB.Bl- Bi-lateral HVC lesion
Pre-lesion
Post-lesion
Left Hemisphere
Type 11
Type 12
Gargle Spectrograms
Type 13
Right Hemisphere
Type 17
Chick-a-dee Spectrograms

B)

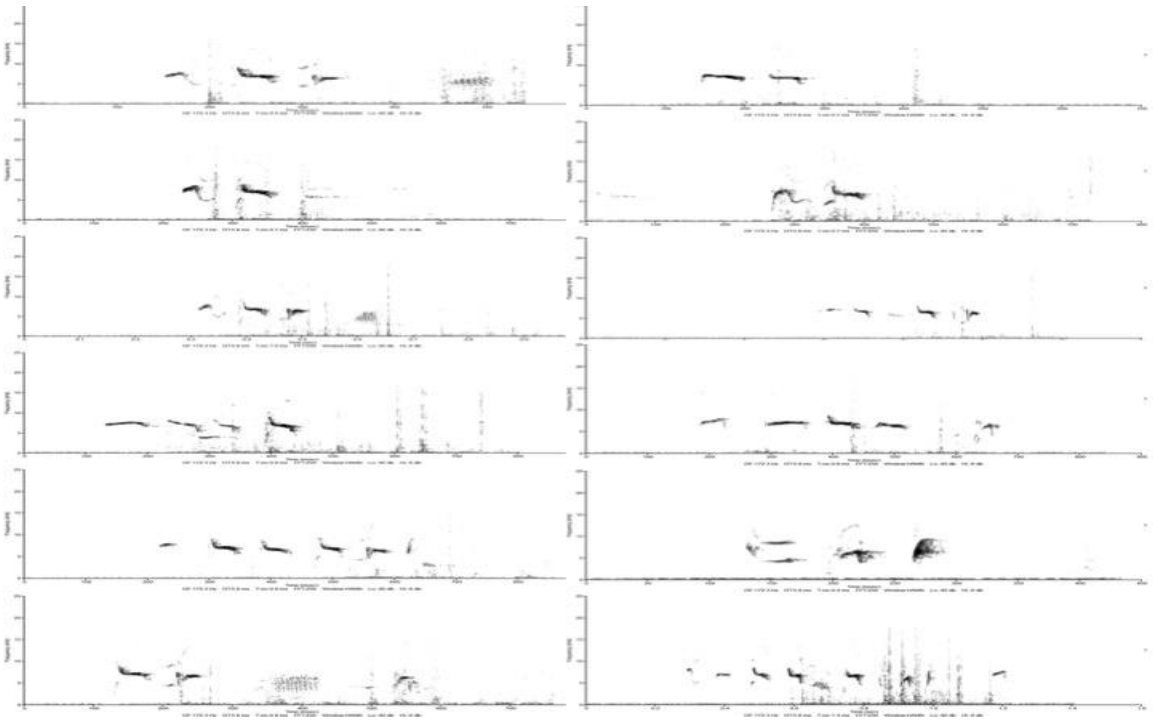


Figure 3- 12 In panel A, diagrams of sagittal sections of the black-capped chickadee, lB.Bl, brain depicting the bilateral HVC lesions in blue and the needle tracks in red. Also included are the spectrograms of the different *gargle* types pre-lesion and post-lesion. Also at the bottom of this figure are spectrograms of an example of a pre- and post-lesion *chick-a-dee* call. Panel B there are sample spectrograms of all the variable *gargle* type calls produced post-lesion that could not be classified and compared to *gargle* calls pre-lesion.

Call Type 11. Call type 11 was measured for the bioacoustic properties of each individual note as illustrated in Figure 13-13 (see Appendix A). Following the lesion this call type was 43% shorter in duration. Notes 3 and 5 had reduced acoustic complexity with lower harmonic structure visible in the spectrogram and significantly changed frequency measures.

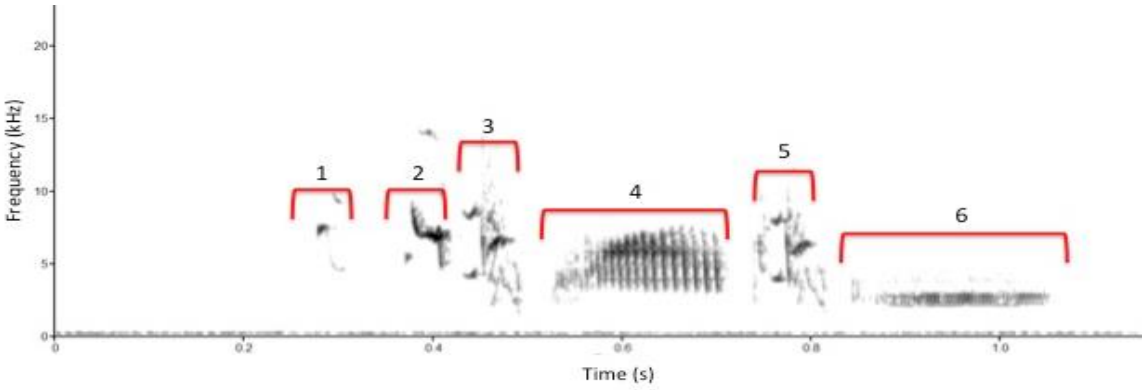


Figure 3- 13 Spectrogram showing where the individual notes are designated in call type 11 for bird lB.Bl. The *gargle* call is composed of 6 notes, indicated here. Time is depicted on the x-axis and frequency (in Hz) is depicted on the y-axis.

Call Type 12. Call type 11 was measured for the bioacoustic properties of each individual note as illustrated in Figure 3-14. Although bioacoustic differences were evident post-lesion (see Figure 3-12), a huge amount of variability in the measures resulted in non-significant statistical differences for many of the notes (see Appendix A). Notes 1 and 2 were significantly shorter in duration and note 3 had significant changes in frequency measures. Although not significant based on my measures, there was also an apparent reduction in acoustic complexity of note 3.

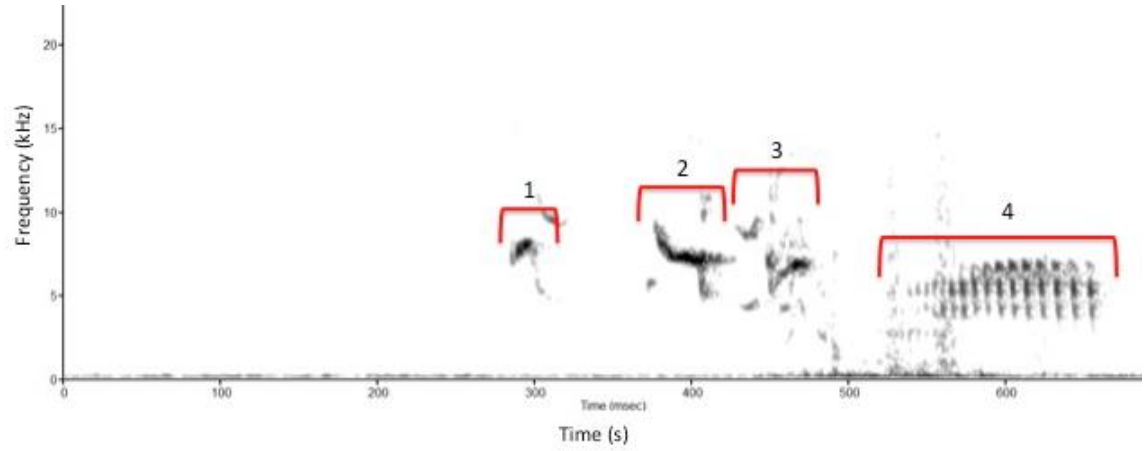


Figure 3- 14 Spectrogram showing where the individual notes are designated in call type 12 for bird lB.Bl. The *gargle* call is composed of 4 notes, indicated here. Time is depicted on the x-axis and frequency (in Hz) is depicted on the y-axis.

Call Type 13. Call type 13 was measured for the bioacoustic properties of each individual note as illustrated in Figure 3-15. The sample size for this *gargle* call type was very small (pre-lesion n = 3, post-lesion n = 1) so the statistical analyses should be interpreted with extreme caution. In general Notes 3 and 5 appeared to drastically reduce in their acoustic structure and note 4 was almost unrecognizable in the spectrogram.

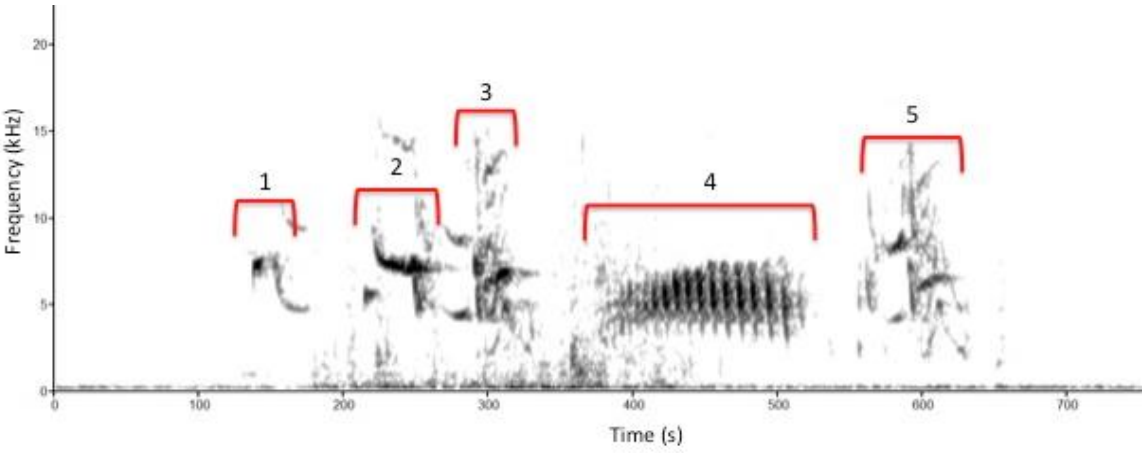


Figure 3- 15 Spectrogram showing where the individual notes are designated in call type 13 for bird lB.Bl. The *gargle* call is composed of 5 notes, indicated here. Time is depicted on the x-axis and frequency (in Hz) is depicted on the y-axis.

Call Type 17. Call type 17 was measured for the bioacoustic properties of each individual note (see Figure 3-16). Following the lesion this call type was 40% shorter in duration. Notes 1, 3 and 4 had reduced acoustic complexity with lower harmonic structure visible in the spectrogram and significantly changed frequency measures.

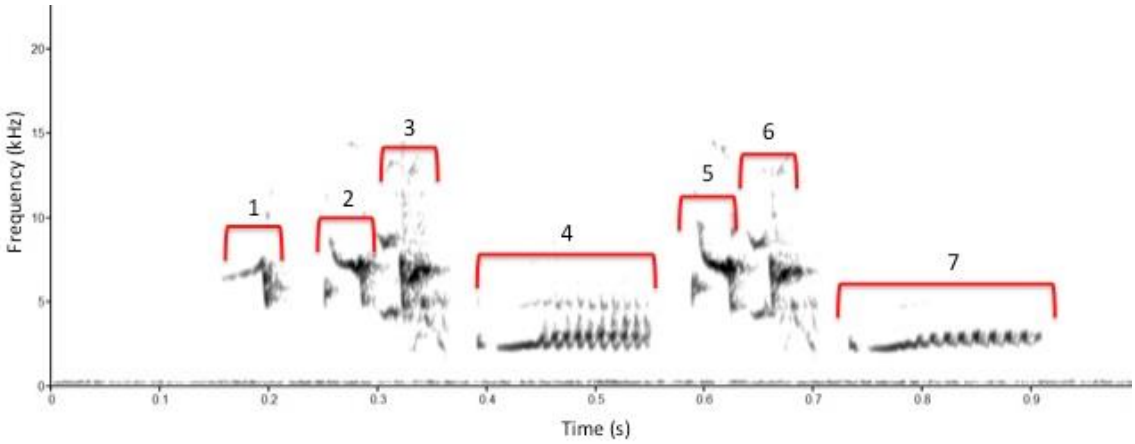
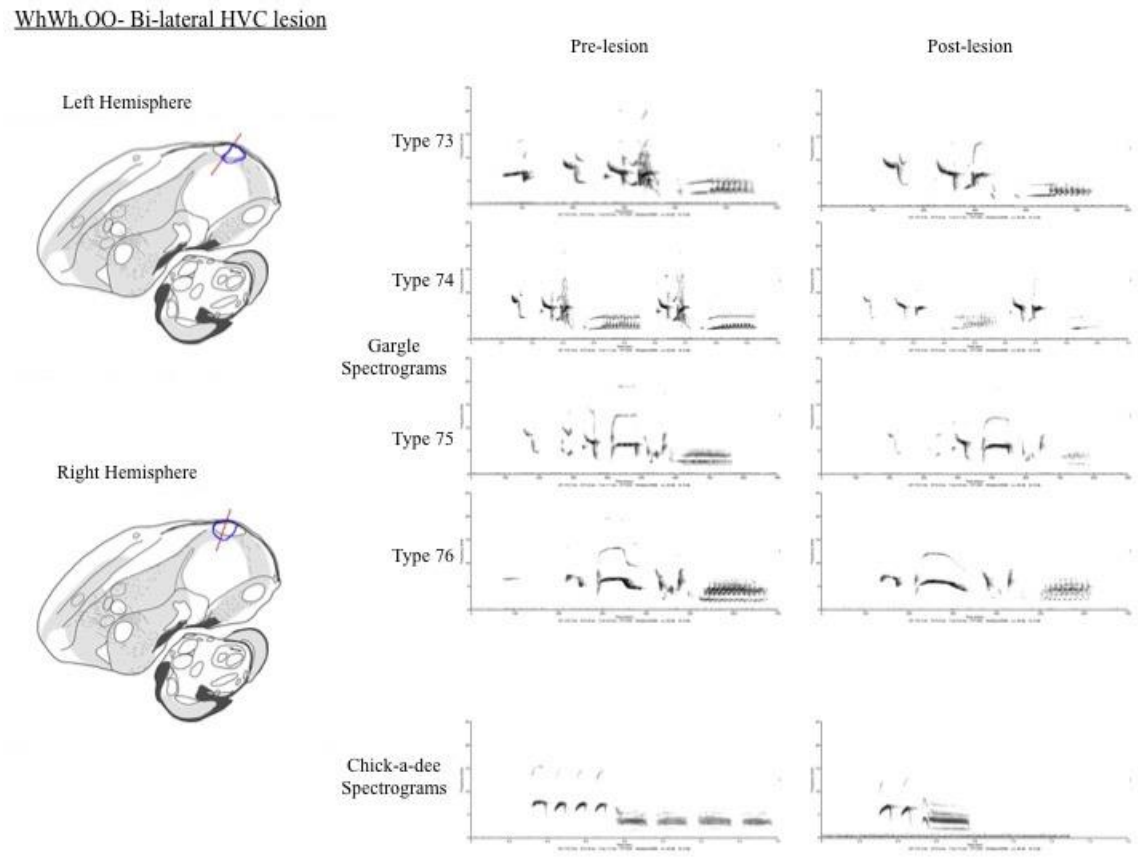


Figure 3- 16 Spectrogram showing where the individual notes are designated in call type 17 for bird lB.Bl. The *gargle* call is composed of 7 notes, indicated here. Time is depicted on the x-axis and frequency (in Hz) is depicted on the y-axis.

3.3.1.1.2 Bird WhWh.OO

The lesion locations and example spectrograms of pre- and post-lesion calls are presented in Figure 3-17. This bird had lesions that damaged HVC in both hemispheres. Prior to lesions it produced 4 *gargle* call types, and post-lesion it produced 4 of those as well as a larger number unidentifiable calls (Figure 3-17, panel B). *Gargle* calls that were identified post-lesion generally had less complex acoustic structure with reduced harmonic structure in several notes (see Figure 3-17, panel A). Detailed examples are provided below and complete statistical comparisons of acoustic measures are provided in Appendix A.

A)


WhWh.OO- Bi-lateral HVC lesion
Pre-lesion
Post-lesion
Left Hemisphere
Type 73
Type 74
Gargle Spectrograms
Type 75
Right Hemisphere
Type 76
Chick-a-dee Spectrograms

B)

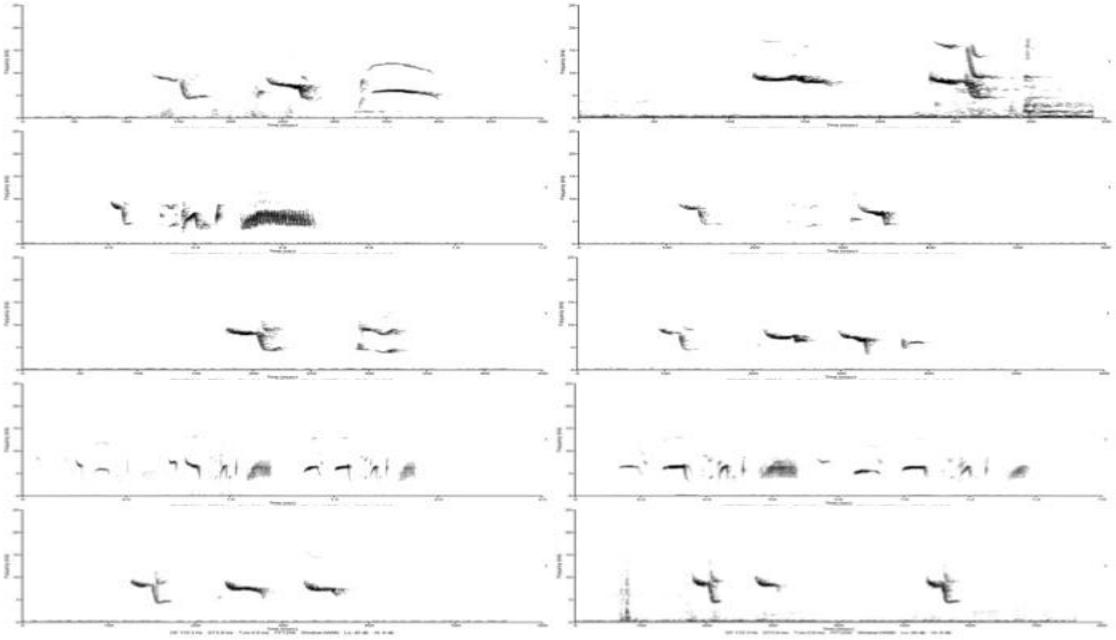


Figure 3- 17 In panel A, diagrams of sagittal sections of the black-capped chickadee, WhWh.OO, brain depicting the bilateral HVC lesions in blue and the needle tracks in red. Also included are the spectrograms of the different *gargle* types pre-lesion and post-lesion. Also at the bottom of this figure are the spectrograms of an example of a pre- and post-lesion *chick-a-dee* call. In panel B there are sample spectrograms of all the variable *gargle* type calls produced post-lesion that could not be classified and compared to *gargle* calls pre-lesion.

Call Type 73. Call type 73 was measured for the bioacoustic properties of each individual note as illustrated in Figure 3-18. Following the lesion this call type was 21% shorter in duration. Notes 4 and 5 had reduced acoustic complexity with lower harmonic structure visible in the spectrogram and significantly changed frequency measures.

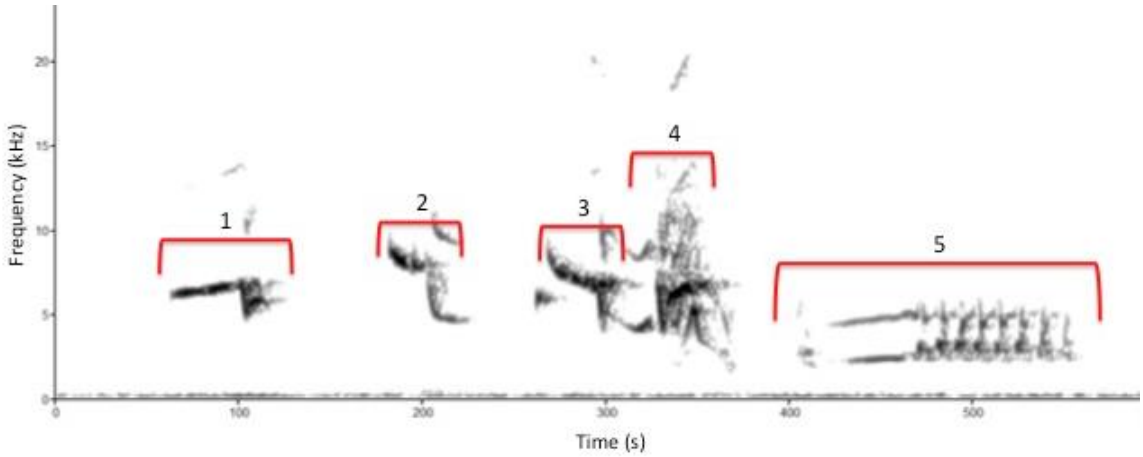


Figure 3- 18 Spectrogram showing where the individual notes are designated in call type 73 for bird WhWh.OO. The *gargle* call is composed of 5 notes, indicated here. Time is depicted on the x-axis and frequency (in Hz) is depicted on the y-axis.

Call Type 74. Call type 74 was measured for the bioacoustic properties of each individual note as illustrated in Figure 3-19. Following the lesion note 3 was 16% shorter, and note 7 was 37% shorter. Notes 3, 6 and 7 had reduced acoustic complexity with lower harmonic structure visible in the spectrogram and significantly changed frequency measures.

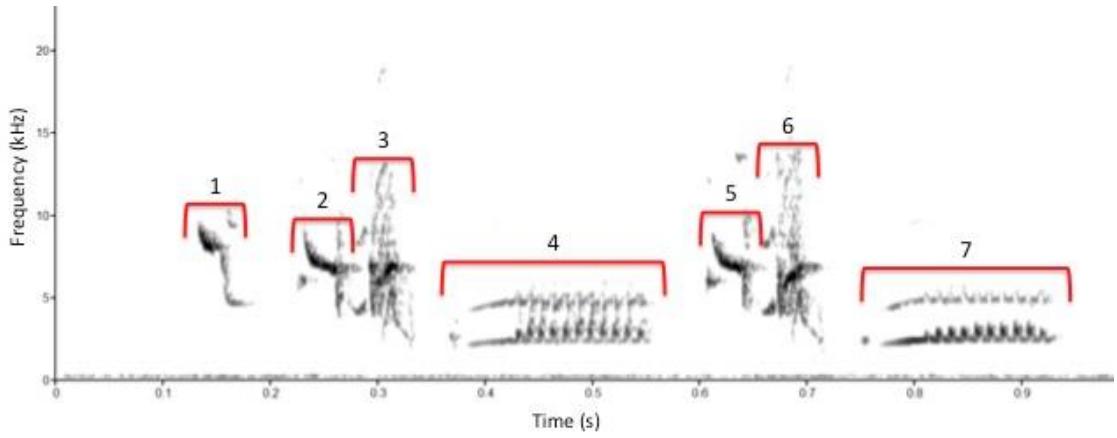


Figure 3- 19 Spectrogram showing where the individual notes are designated in call type 74 for bird WhWh.OO. The *gargle* call is composed of 7 notes, indicated here. Time is depicted on the x-axis and frequency (in Hz) is depicted on the y-axis.

Call Type 75. Call type 75 was measured for the bioacoustic properties of each individual note as illustrated in Figure 3-20. Following the lesion note 1 was 17% longer, and note 6 was 51% shorter. Note 2, 4, 5 and 6 had reduced acoustic complexity with lower harmonic structure visible in the spectrogram and significantly changed frequency measures.

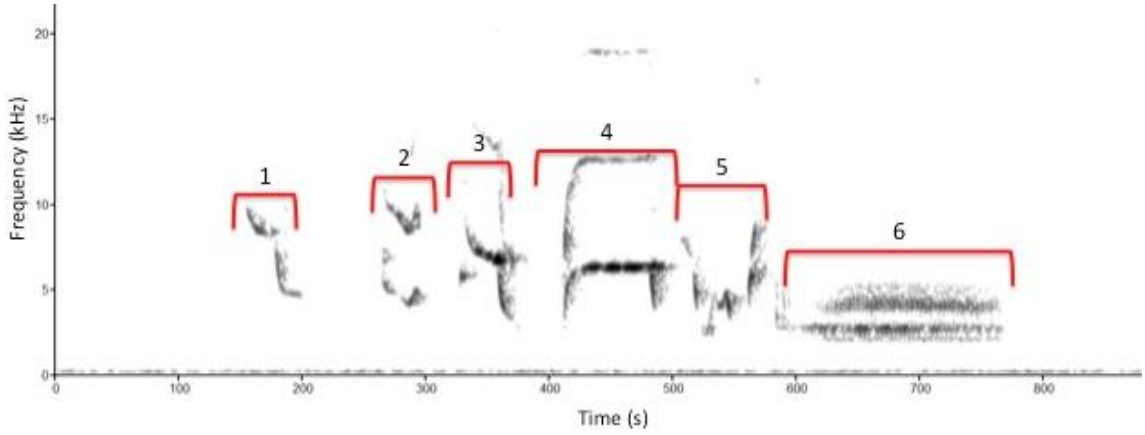


Figure 3- 20 Spectrogram showing where the individual notes are designated in call type 75 for bird WhWh.OO. The *gargle* call is composed of 6 notes, indicated here. Time is depicted on the x-axis and frequency (in Hz) is depicted on the y-axis.

Call Type 76. Call type 76 was measured for the bioacoustic properties of each individual note as illustrated in Figure 3-21. Following the lesion notes 2, 3, and 4 were a bit longer, and note 5 was 22% shorter. Note 2, 3, 4 and 5 had reduced acoustic complexity with lower harmonic structure visible in the spectrogram and significantly changed frequency measures.

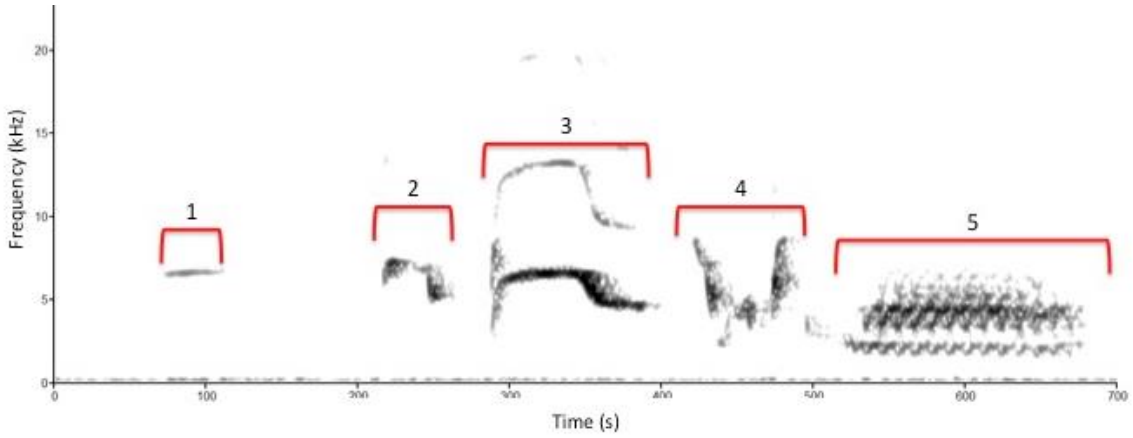
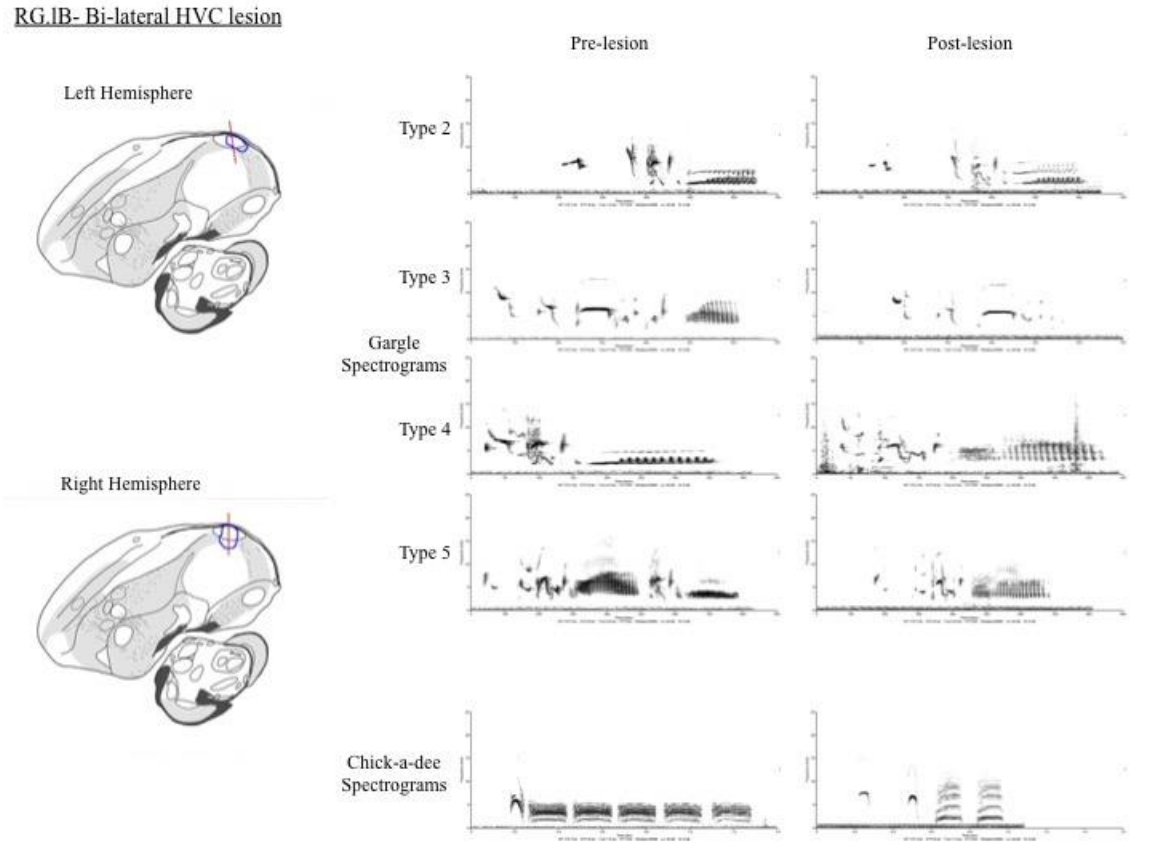


Figure 3- 21 Spectrogram showing where the individual notes are designated in call type 76 for bird WhWh.OO. The *gargle* call is composed of 5 notes, indicated here. Time is depicted on the x-axis and frequency (in Hz) is depicted on the y-axis.

3.3.1.1.3 Bird RG.lB

The lesion locations and example spectrograms of pre- and post-lesion calls are presented in Figure 3-22. This bird had lesions that damaged HVC in both hemispheres. Prior to lesions it produced 4 *gargle* call types, and post-lesion it produced 4 of those as well as a number unidentifiable calls (Figure 3-22, panel B). *Gargle* calls that were identified post-lesion generally had less complex acoustic structure with reduced harmonic structure in several notes (see Figure 3-22, panel A). Detailed examples are provided below and complete statistical comparisons of acoustic measures are provided in Appendix A.

A)



B)

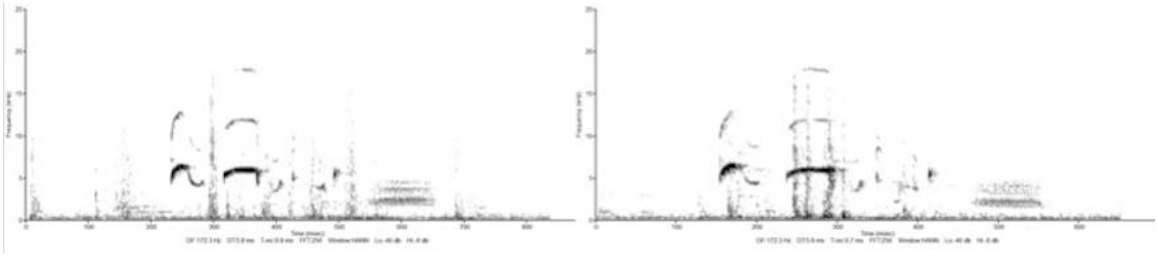


Figure 3- 22 In panel A, diagrams of sagittal sections of the black-capped chickadee, RG.lB, brain depicting the bilateral HVC lesions in blue and the needle tracks in red. Also included are the spectrograms of the different *gargle* types pre-lesion and post-lesion. Also at the bottom of this figure are the spectrograms of an example of a pre- and post-lesion *chick-a-dee* call. In panel B there are sample spectrograms of all the variable *gargle* type calls produced post-lesion that could not be classified and compared to *gargle* calls pre-lesion.

Call Type 2. Call type 2 was measured for the bioacoustic properties of each individual note as illustrated in Figure 3-23. Following the lesion notes 2 was 16% shorter, and note 4 was 35% shorter. Note 2 and 4 had reduced acoustic complexity with lower harmonic structure visible in the spectrogram and significantly changed frequency measures. Note 3 also had two significant changes in frequency measures.

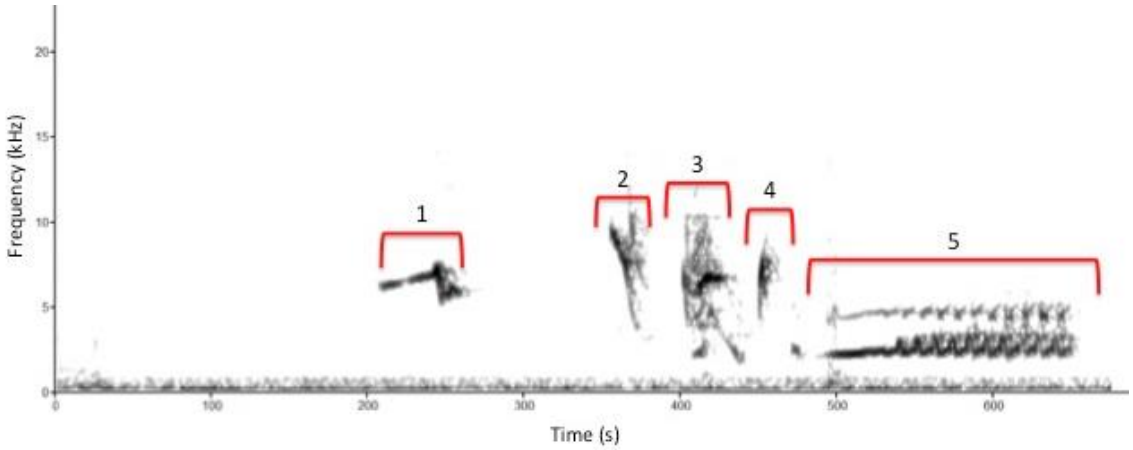


Figure 3- 23 Spectrogram showing where the individual notes are designated in call type 2 for bird RG.lB. The *gargle* call is composed of 5 notes, indicated here. Time is depicted on the x-axis and frequency (in Hz) is depicted on the y-axis.

Call Type 3. Call type 3 was measured for the bioacoustic properties of each individual note as illustrated in Figure 3-24. Following the lesion the overall call was 11% longer, however note 4 was 51% longer, and note 5 was 36% longer. Notes 1, 3, and 6 had reduced acoustic complexity with lower harmonic structure visible in the spectrogram and significantly changed frequency measures. Note 2 also had two significant changes in frequency measures.

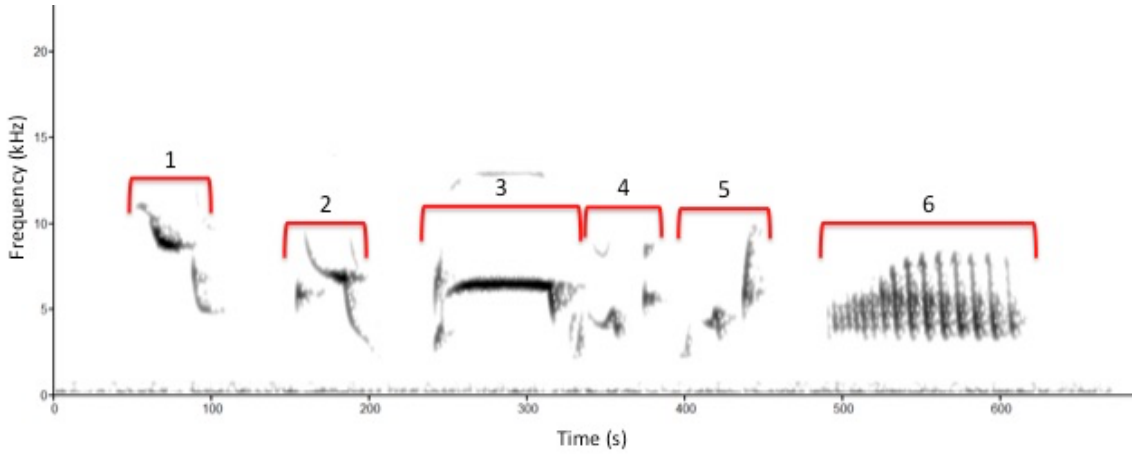


Figure 3- 24 Spectrogram showing where the individual notes are designated in call type 3 for bird RG.lB. The *gargle* call is composed of 6 notes, indicated here. Time is depicted on the x-axis and frequency (in Hz) is depicted on the y-axis.

Call Type 4. Call type 4 was measured for the bioacoustic properties of each individual note as illustrated in Figure 3-25. Following the lesion note 1 was 12% shorter, and note 4 was 34% shorter. Notes 2 and 4 had reduced acoustic complexity with lower harmonic structure visible in the spectrogram and significantly changed frequency measures.

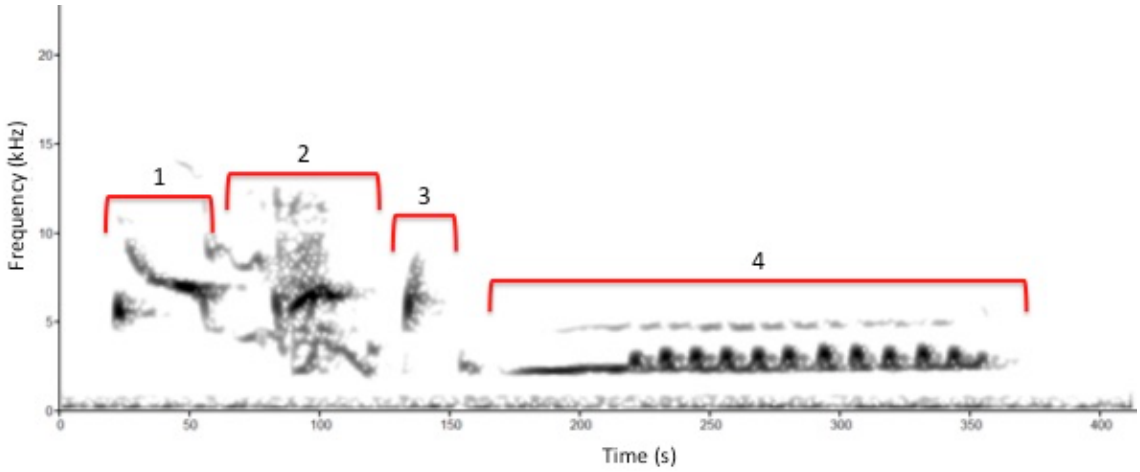


Figure 3- 25 Spectrogram showing where the individual notes are designated in call type 4 for bird RG.lB. The *gargle* call is composed of 4 notes, indicated here. Time is depicted on the x-axis and frequency (in Hz) is depicted on the y-axis.

Call Type 5. Call type 5 was measured for the bioacoustic properties of each individual note as illustrated in Figure 3-26. Notes 2, 3, 5 and 6 had reduced acoustic complexity with lower harmonic structure visible in the spectrogram and significantly changed frequency measures. Although not significant, there was a large reduction in the maximal frequency and the note peak frequency for note 8.

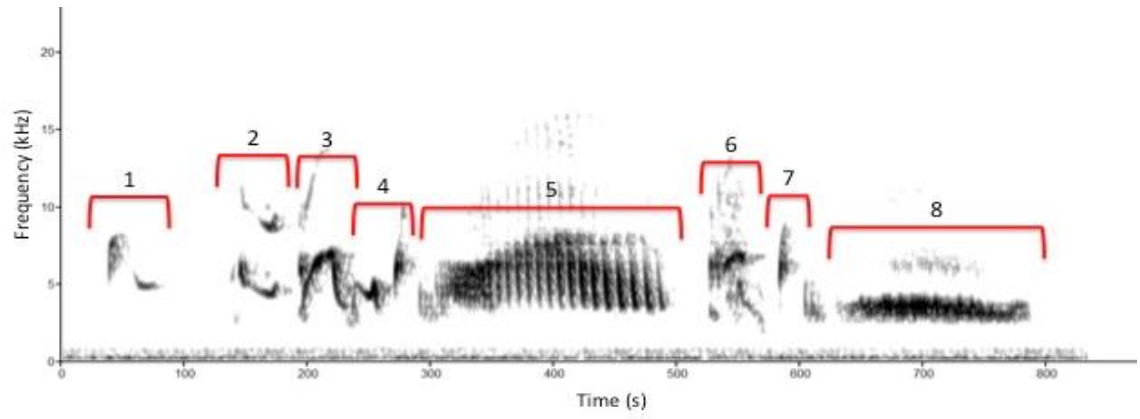


Figure 3- 26 Spectrogram showing where the individual notes are designated in call type 5 for bird RG.lB. The *gargle* call is composed of 8 notes, indicated here. Time is depicted on the x-axis and frequency (in Hz) is depicted on the y-axis.

3.3.1.1.4 Bird GrPe.O

The lesion locations and example spectrograms of pre- and post-lesion calls are presented in Figure 3-27. This bird had lesions that damaged HVC in both hemispheres. Prior to lesions it produced 1 *gargle* call type, and post-lesion it produced this same *gargle* type. *Gargle* calls that were identified post-lesion generally had a similar structure, however the harmonic structure of the notes was simpler (see Figure 3-27). Detailed examples are provided below and complete statistical comparisons of acoustic measures are provided in Appendix A.

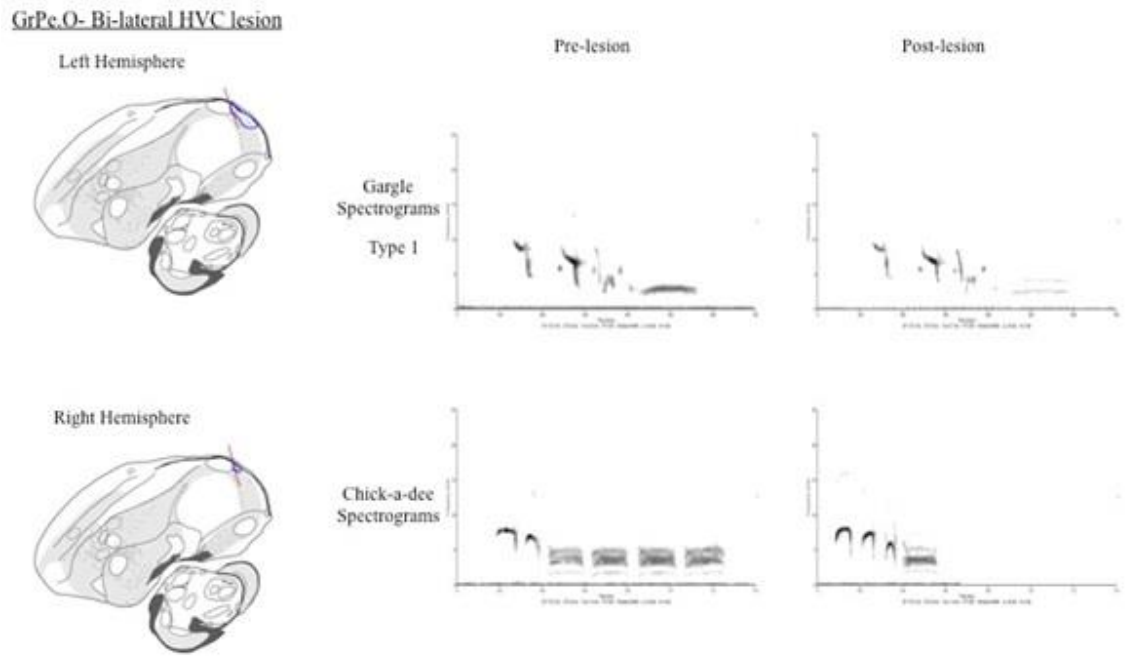


Figure 3- 27 Diagrams of sagittal sections of the black-capped chickadee, GrPe.O, brain depicting the bilateral HVC lesions in blue and the needle tracks in red. Also included are the spectrograms depicting the *gargle* type pre-lesion and post-lesion. Also at the bottom of this figure are the spectrograms showing an example of a pre- and post-lesion *chick-a-dee* call.

Call Type 1. Call type 1 was measured for the bioacoustic properties of each individual note as illustrated in Figure 3-28. Notes 2 and 4 had reduced acoustic

complexity with lower harmonic structure visible in the spectrogram and significantly changed frequency measures.

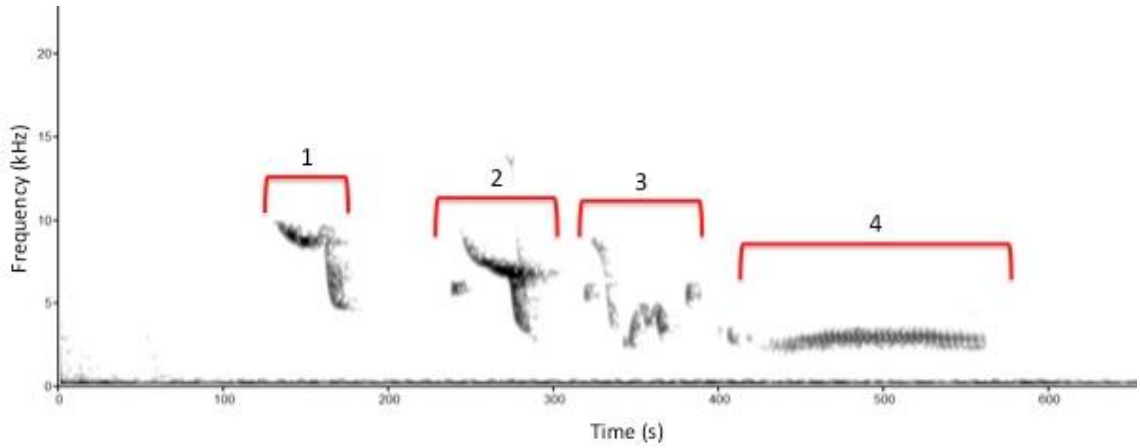


Figure 3- 28 Spectrogram showing where the individual notes are designated in call type 1 for bird GrPe.O. The *gargle* call is composed of 4 notes, indicated here. Time is depicted on the x-axis and frequency (in Hz) is depicted on the y-axis.

3.3.1.2 Missed lesioned birds

Birds Br.O and BGr.Y were considered missed lesioned birds, where the same surgical procedure was conducted, and the ibotenic acid was injected missed HVC entirely in the left and right hemispheres. Overall, the missed lesioned bird Br.O showed little effect of the lesion on the *gargle* call. The measures that did differ post-lesion did not have very large effect sizes. Whereas for bird BGr.Y there were significant differences in the *gargle* call after the lesion, however this may be due to the missed lesion in one hemisphere being in cerebellum, which is crucial for motor control of vocal production.

3.3.1.2.1 Bird Br.O

The lesion locations and example spectrograms of pre- and post-lesion calls are presented in Figure 3-29. This bird had lesions that did not damage HVC in either hemisphere. In each hemisphere the lesion hit just caudal of HVC (see Figure 3-29). Prior to lesions it produced 2 *gargle* call types, and post-lesion it produced both of those. *Gargle* calls that were identified post-lesion generally had a similar structure, and note composition, and did not differ greatly on the spectrograms (see Figure 3-29). Detailed examples are provided below and complete statistical comparisons of acoustic measures are provided in Appendix A.

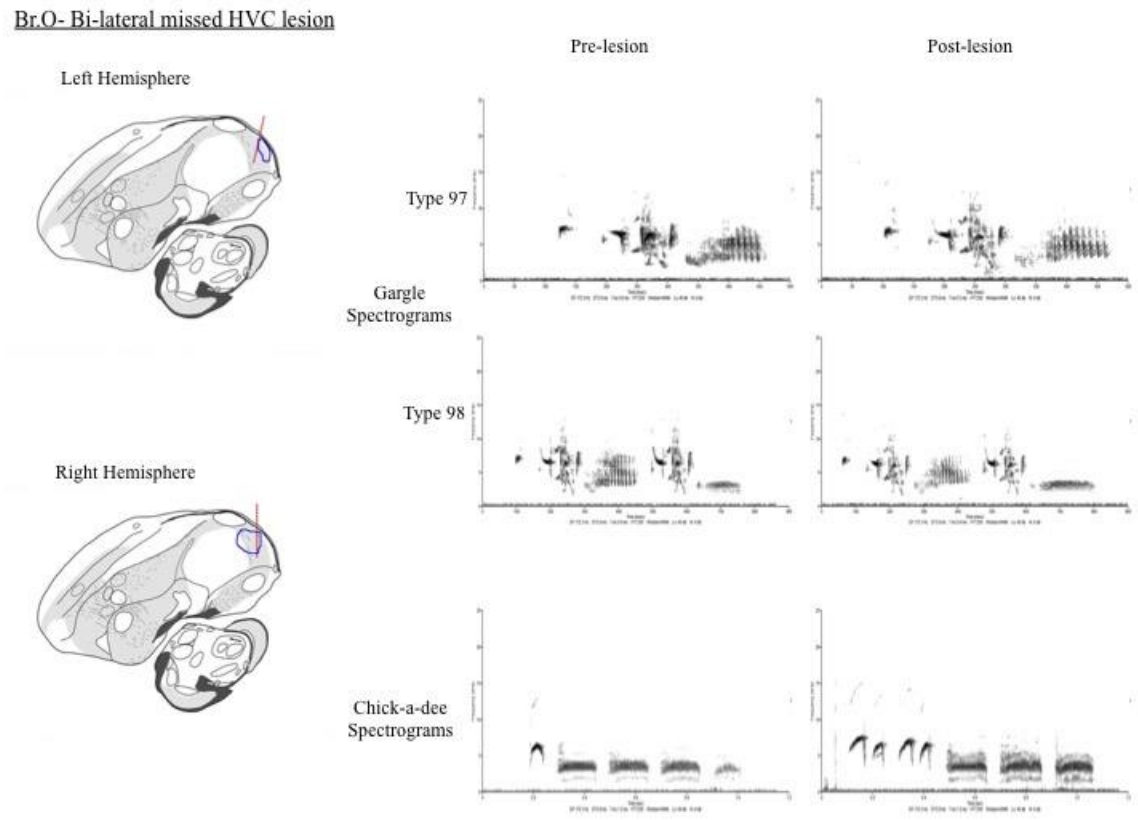


Figure 3- 29 Diagrams of sagittal sections of the black-capped chickadee, Br.O, brain depicting the bilateral HVC lesions in blue and the needle tracks in red. Also

included are the spectrograms of the different *gargle* types pre-lesion and post-lesion. Also at the bottom of this figure are the spectrograms of an example of a pre- and post-lesion *chick-a-dee* call.

Call Type 97. Call type 97 was measured for the bioacoustic properties of each individual note as illustrated in Figure 3-30. No differences were observable from the spectrograms and very little differed statistically for the individual notes' bioacoustic measures.

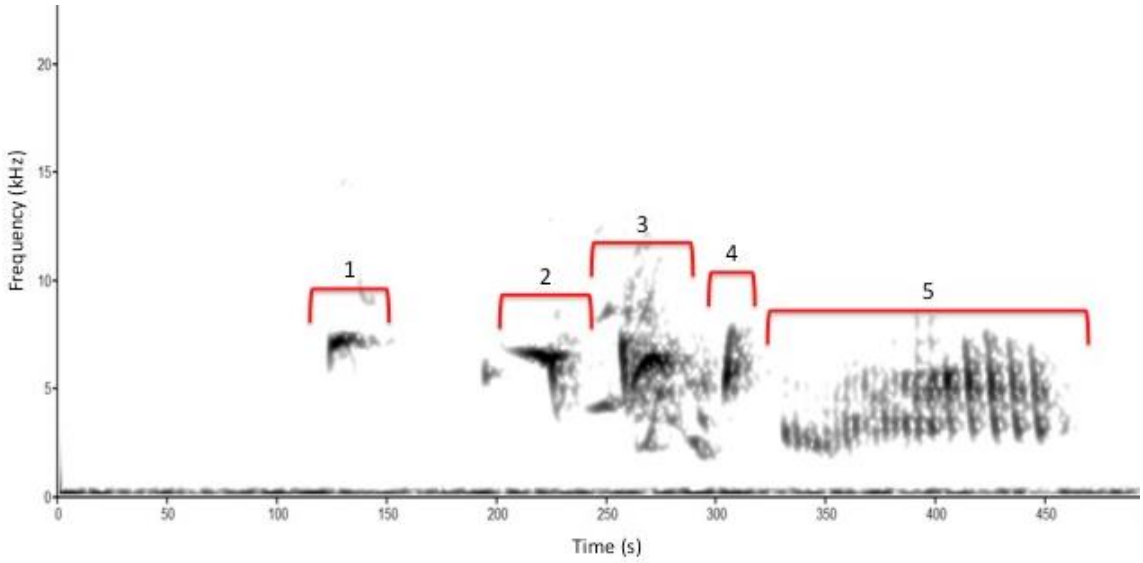


Figure 3- 30 Spectrogram showing where the individual notes are designated in call type 97 for bird Br.O. The *gargle* call is composed of 5 notes, indicated here. Time is depicted on the x-axis and frequency (in Hz) is depicted on the y-axis.

Call Type 98. Call type 98 was measured for the bioacoustic properties of each individual note as illustrated in Figure 3-31. No differences were observable visually from the spectrograms. However, there were a number of significant differences in the measures for notes 1, 2, 3, 5, 6, 7, and 9, most of which were significant decreases in SF, EF, PF, TF, and F_{max} post-lesion, whereas f_0 and NPF significantly increased post-lesion. These significant changes had relatively small effect sizes.

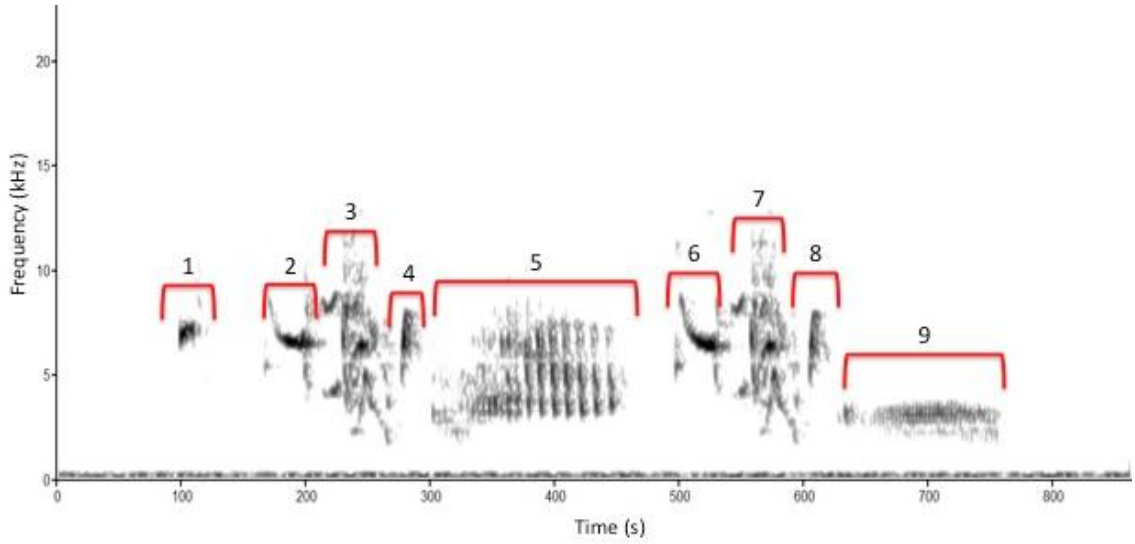


Figure 3- 31 Spectrogram showing where the individual notes are designated in call type 98 for bird Br.O. The *gargle* call is composed of 9 notes, indicated here. Time is depicted on the x-axis and frequency (in Hz) is depicted on the y-axis.

3.3.1.2.2 Bird BGr.Y

The lesion locations and example spectrograms of pre- and post-lesion calls are presented in Figure 3-32. This bird had lesions that did not damage HVC in either hemisphere, however one of the lesions entered the cerebellum. In each hemisphere the lesion hit just caudal of HVC (see Figure 3-29). Prior to lesions it produced 1 *gargle* call type, and post-lesion it produced the same one. *Gargle* calls that were identified post-lesion generally had a similar structure, and note composition, and did not differ greatly when examining the spectrograms (see Figure 3-32). Detailed examples are provided below and complete statistical comparisons of acoustic measures are provided in Appendix A.

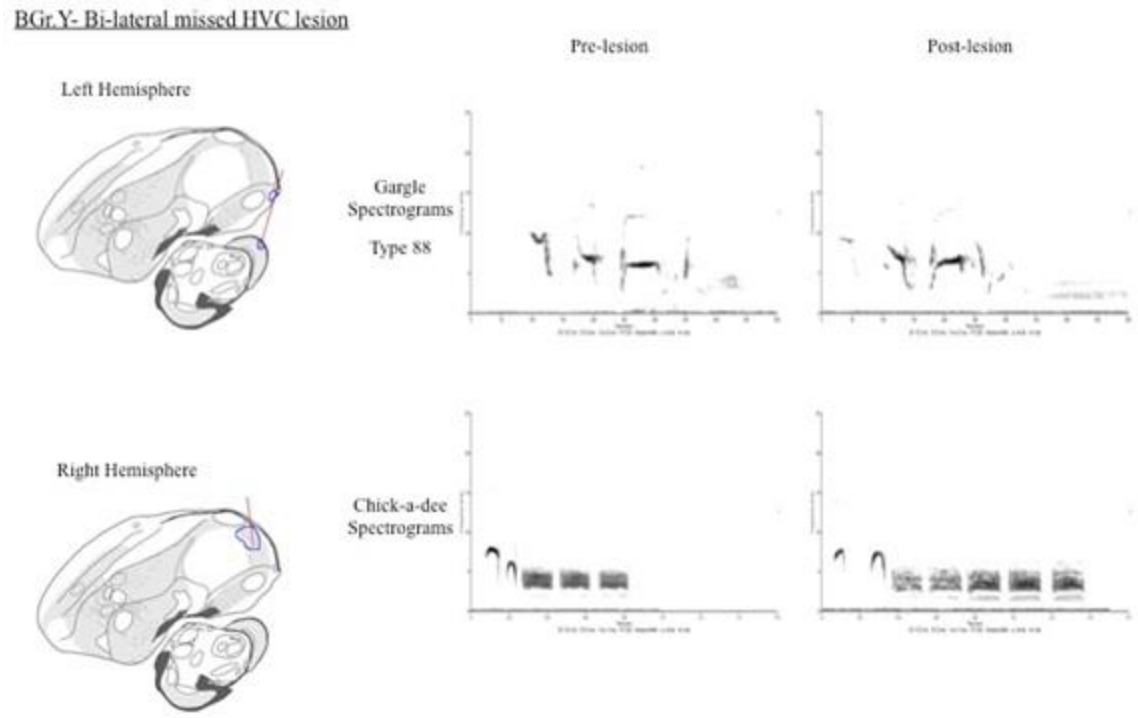


Figure 3- 32 Diagrams of sagittal sections of the black-capped chickadee, BGr.Y, brain depicting the bilateral HVC lesions in blue and the needle tracks in red. Also included are the spectrograms of the *gargle* type pre-lesion and post-lesion. Also at the bottom of this figure are the spectrograms of an example of a pre- and post-lesion *chick-a-dee* call.

Call Type 88. Call type 88 was measured for the bioacoustic properties of each individual note as illustrated in Figure 3-33. The call was 30% longer post-lesion, where note 4 was 69% longer, and note 5 was 123% longer. After the lesion, note 1 and note 2 flattened out in the top portion of the note, note 3 became more angled instead of being straight across and note 4 became a mirror image of itself (see Figure 3-33). Possibly due to the damage to the cerebellum, notes 1, 3 and 5 had reduced acoustic complexity with lower harmonic structure visible in the spectrogram and significantly changed frequency measures.

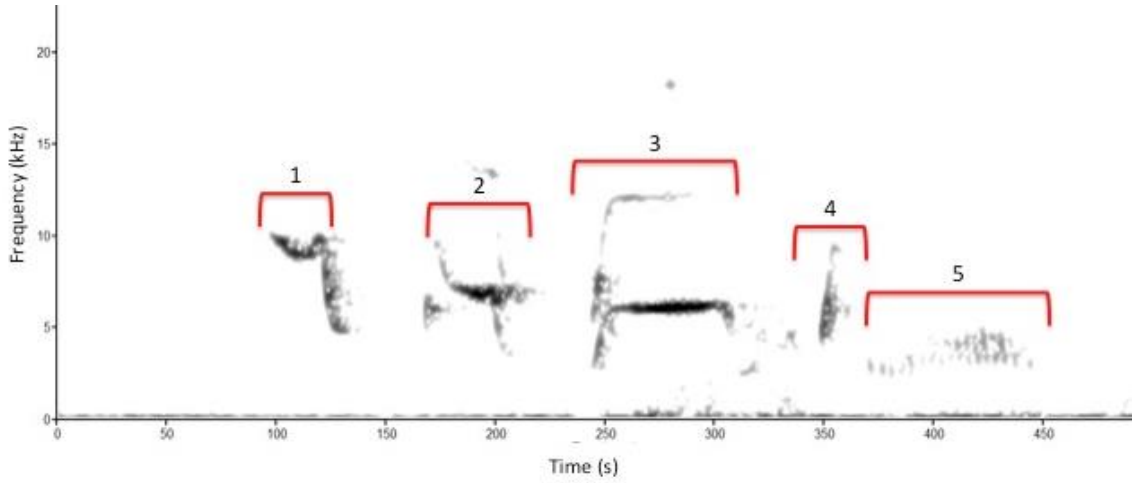


Figure 3- 33 Spectrogram showing where the individual notes are designated in call type 88 for bird BGr.Y. The *gargle* call is composed of 5 notes, indicated here. Time is depicted on the x-axis and frequency (in Hz) is depicted on the y-axis.

3.3.2 *Chick-a-dee* calls

Overall, after the bilateral HVC lesions the *chick-a-dee* calls changed somewhat. The D notes' spectrograms were much more varied post-lesion, and they tended to span a greater frequency range. If the birds did not produce D-hybrid notes, which are when an A, B or C note attaches itself to a D note, they produced them post-lesion and vice versa. There were also fewer D notes produced post-lesion, which were also usually longer in duration. For the control birds, the overall *chick-a-dee* calls were longer, which was accounted for by an increased production of D notes post-lesion. There were also changes in the some of the frequency measures of the A notes in the missed lesioned birds. Details for each individual bird are discussed below, and detailed statistical comparisons are shown in Appendix B.

3.3.2.1 Bilateral lesioned birds

3.3.2.1.1 Bird lB.Bl

The *chick-a-dee* call was measured for the bioacoustic properties of each individual note. Overall the *chick-a-dee* calls were 41% shorter, which is accounted for by the overall decrease in D notes produced. The A note was also 41% shorter and the B notes were 33% longer. The A, B and D notes did show structural changes when examining the spectrograms (see Figure 3-12). Notes A had significantly decreased frequency measures. And although not significant, there were increases in the frequency measures for the D notes (see Appendix B).

3.3.2.1.2 Bird WhWh.OO

The *chick-a-dee* call was measured for the bioacoustic properties of each individual note. Overall the *chick-a-dee* calls were 36% shorter, which is accounted for by the overall decrease in D notes produced. The A notes were 33% shorter, and the D notes were 14% longer. There were differences in the spectrogram post-lesion, in particular for the D notes (see Figure 3-17). The A and D notes had significant changes in the frequency measures (see Appendix B).

3.3.2.1.3 Bird RG.lB

The *chick-a-dee* call was measured for the bioacoustic properties of each individual note. Overall the *chick-a-dee* calls were 51% shorter, which is accounted for by the overall decrease in D notes produced. The D notes were seriously affected by the lesion when the spectrograms were examined (see Figure 3-22). The D notes were 20% shorter. There were significant differences in the D notes frequency measures. The other notes were unaffected (see Appendix B).

3.3.2.1.4 Bird GrPe.O

The *chick-a-dee* call was measured for the bioacoustic properties of each individual note. Overall the *chick-a-dee* calls were 52% shorter, which is accounted for by the overall decrease in D notes produced. The D notes were 10% longer post-lesion. The D notes were seriously affected by the lesion when the spectrograms were examined (see Figure 3-27). There were significant differences in the B notes frequency measures (see Appendix B).

3.3.2.2 Missed lesioned birds

3.3.2.2.1 Bird Br.O

The *chick-a-dee* call was measured for the bioacoustic properties of each individual note. Overall the *chick-a-dee* calls were 66% longer, which is accounted for by the overall increase in D notes produced. There were significant differences in the A and B notes frequency measures. And unlike the HVC lesioned birds there were no differences in frequency measure or the spectrograms for the D notes (see Figure 3-29)(see Appendix B).

3.3.2.2.2 Bird Br.O

The *chick-a-dee* call was measured for the bioacoustic properties of each individual note. Similar to the other missed lesioned bird, the overall *chick-a-dee* was 43% longer, which is accounted for by the overall increase in D notes produced. There were no differences between the spectrograms, or for the frequency measures of any of the notes (see Figure 3-32) (see Appendix B).

3.4 Discussion

This study was conducted to determine if HVC was involved in the production of the *chick-a-dee* and the *gargle* calls of the black-capped chickadee, and how these lesions would impact the acoustic structure of these calls. The data support the conclusion that HVC is involved in the production of calls, specifically the *gargle* and *chick-a-dee* calls. However there are a variety of different effects on the *gargle* calls compared to the *chick-a-dee* calls.

3.4.1 *Gargle* calls

In terms of the *gargle* calls, when examining the spectrograms we see that there are effects for birds lB.Bl, WhWh.OO and RG.lB, whereas there is little effect of the HVC lesion on the *garg*le call of bird GrPe.O. The control birds, Br.O and BGr.Y, also show little effect of the missed lesions on the *gargle* call. However, the spectrograms of the *gargle* calls post-lesion were extremely variable for birds with the bilateral HVC lesions. Not only were the structures of the identifiable calls affected, but there were a number of vocalizations produced post-lesion that were comprised of *gargle* note types, but did not match any of the *gargles* produced pre-lesion (see Figures 3-12, 3-17 and 3-22; B panels). These unidentified calls were produced in three of the four successfully HVC lesioned birds, and did not occur in either missed lesion birds. The *gargle* call in free-living birds is produced in a stereotyped manner, where the production of the *gargle* is consistent upon subsequent vocalizations (Baker, Tracy, & Miyasato, 1996, Otter, 2007). Therefore, the variability I observed post-lesion is atypical for *gargle* call production. Similarly, zebra finches with damage HVC lose stereotyped song parameters

(Scharff, Kirn, Grossman, Macklis, & Nottebohm, 2000; Simpson & Vicario, 1990; Thompson & Johnson, 2005; Williams & McKibben, 1992).

Zebra finches also produce a long-call when they are placed in visual isolation from one another. This call is sexually dimorphic: the male call has more complex acoustic features than the female long-call (Price, 1979; Zann, 1984, 1985). Not only is the male call more complex, it is also learned in a similar way to how birds learn their song, whereas the female call is innate (Zann, 1985). Bilaterally HVC-lesioned male zebra finches had altered male long-calls, however females with the same type of lesion had intact long-calls (Simpson & Vicario, 1990). These lesions affected the more complex and learned male long-call, just like the bilateral HVC lesions affected the *gargle* calls in my study. Although there is some evidence suggesting that the *gargle* call is learned in chickadees, the fact that it is affected similarly to song and the male long-call in zebra finches that have HVC lesions would suggest that this call is at least partially learned (Baker et al., 2000; Thompson & Johnson, 2005).

The bioacoustic analysis results also indicated some overarching similarities in defects in the *gargle* calls post-lesion. For example, the notes with complex harmonic structure observed in many of the different *gargle* types (e.g., notes 3 and 5 in Figure 3-13), were the most seriously affected post-lesion. However, these types of notes were not affected by lesion in missed lesion bird Br.O. These types of notes showed similar effects of HVC lesion across the different birds, with an increased end frequency, decreased top frequency, decreased peak frequency, and decreased loudest frequency (F_{max}) (see Appendix A). However, these bioacoustic changes do not account for all of the structural changes, such as the decreased complex harmonic structure that occurs on these note

types post-lesion. Also, the huge amount of variability observed in the notes post-lesion make it difficult to find statistical differences. In zebra finches with single right hemisphere HVC lesions, there is a decrease in the top frequency, whereas left hemisphere lesions increased the lowest frequency (Halle et al., 2003). Similarly, bilateral HVC lesions in zebra finches, turn the male long-call into an innate female long-call, where all the complex parts of the call are lost (Simpson & Vicario, 1990). Therefore, it is plausible that the *gargle* calls obtained post bilateral HVC lesions are the innate portions of the call, as they do seem acoustically simpler than those pre-lesion. The aspects of the calls that are lost due to the lesions could be the portions of the calls that are learned, which would explain why we see differences in the *gargle* call across different geographic regions (Baker et al., 2000)

There were very few effects of HVC lesions in bird GrPe.O; the lesion for this bird could be less detrimental, and affected less of HVC in each hemisphere. HVC has projections to multiple structures, and variety of different neuron types. HVC serves different purposes depending on the neuron type that is involved, it plays both a primary role in song learning early on in life, and these neuron types project to nucleus avalanche, or another neuron type is crucial for song production in adulthood, and project to area X and the robust nucleus of the arcopallium (Roberts et al., 2017). This could potentially account for the small number of differences observed in the *gargle* call for bird GrPe.O. Although it can't be verified, it could be possible that the ibotenic acid reached one type of neuron and not the other, and therefore did not have significant detrimental effects on the *gargle* call, compared to the other 3 bilaterally HVC lesioned birds.

3.4.2 *Chick-a-dee* calls

In terms of the *chick-a-dee* calls, when examining the spectrograms we see that there were effects of HVC lesions for birds lB.Bl, WhWh.OO and RG.lB, whereas there was little effect of the HVC lesion on the *chick-a-dee* call for birds GrPe.O, Br.O and BGr.Y. There was an increased presence of "d-hybrid" notes post-lesion, which are characterized as either an A, B or C type note attached to a D note (Campbell, Hahn, Congdon, & Sturdy, 2016). These notes do occur in intact chickadees; however in the current study if these notes were produced pre-lesion, they were not produced post-lesion and vice-versa. C notes were also extremely uncommon in the experiment. A and B notes were present in relatively equal frequencies pre and post-lesion, whereas there were fewer D notes post-lesion for birds lB.Bl, WhWh.OO, RG.lB and GrPe.O. In addition to these changes in the number of note types produced, there were great changes in the acoustic structure in the *chick-a-dee* calls post-lesion, where the D notes are more varied, especially for birds lB.Bl and RG.lB (see Figures 3-12, 22, Panel A). The missed lesioned birds also had longer *chick-a-dee* calls post-lesion, where there were more D notes produced post-lesion. This is contrary to the findings in the HVC lesioned birds.

Comparing the bioacoustic measures pre- and post-lesion, there are varied effects overall for the missed lesion birds. Although the lesioned HVC birds had a mostly consistent effect on some of the frequency measures of the D notes, upon closer examination of the bioacoustic measures of the missed lesion group, there were no major changes between the *chick-a-dee* calls of missed lesion bird BGr.Y pre to post-lesion. However missed lesion bird Br.O had some effects for note the A note post-lesion: there was a decrease in start and end frequency. For note B, a decrease in start and end

frequencies, as well as in maximal frequency, which are similar to the changes seen in HVC lesioned birds WhWh.OO, and lB.Bl. Therefore, the effects on the missed lesion birds overall are mixed, but there are distinct differences between the HVC lesioned birds and missed lesion birds, where missed lesion birds have longer *chick-a-dee* calls overall post-lesion, which is not observed in the HVC lesioned birds post-lesion, indicating that HVC may be critical for appropriate D note production.

There is evidence that the *chick-a-dee* call is learned, however my results do not support the idea that just the B and C notes are learned and depend on HVC for their production. Rather, my data would suggest that some properties of all notes are learned (Baker et al., 2003; Hughes et al., 1998). It would seem as though for black-capped chickadees, at least some properties of each note are learned, although the notes are still identifiable as either A, B or D notes. These results are similar to the finding in zebra finches that HVC is crucial for the production of the male long-call (Catchpole & Slater, 2008; Kroodsma & Miller, 1996; Marler, 2004; Simpson & Vicario, 1990). HVC is crucial for learning and producing the male typical characteristics of the long-call. When HVC is inactivated, the long-call reverts back to a female typical long-call, which is an innate vocalization. Although the *chick-a-dee* call is still able to be identified post-lesion and is produced in the same syntactic order, the *chick-a-dee* call has some acoustic structure that may be innate and not require HVC, but to modify those note structures based on vocal input may require learning and a functional HVC.

Electrophysiological studies have shown that particular neurons fire in tune with the temporal cues of zebra finch song. It would be of value to investigate if the same is true in chickadees when presented with their more complex calls, the *chick-a-dee* and

gargle calls. (Theunissen & Doupe, 1998). In terms of truly understanding the function of the neurons within HVC, in vivo-electrophysiological studies would be invaluable. Recording freely moving chickadees when they produce their different vocalizations could give us true insight into the role of HVC in these call productions. However, the proposed technology for this has only recently been developed and is currently only used in zebra finches; it would have to be adapted for chickadees, which are on average much smaller (Danish, Aronov, & Fee, 2017; Lynch, Okubo, Hanuschkin, Hahnloser, & Fee, 2016; Okubo, Mackevicius, & Fee, 2014)

3.4.3 Conclusions

The results of this lesion study indicate that HVC is involved in the production of the *chick-a-dee* and *gargle* calls in chickadees, and the effects of HVC lesion seem to be more prominent for the *gargle* calls. This could be because the *gargle* call is more complex acoustically and/or because production of the *gargle* depends more on imitative vocal learning. Further research would be required to explore these possibilities. In Chapter 2 I found that the *gargle* call compared to the *chick-a-dee* call elicited more ZENK-ir in HVC, which suggested that HVC is more active during *gargle* production than *chick-a-dee* production. My lesion results corroborate these findings. Although more work is required to understand the fine details of how the different neural populations in HVC are involved in the production of these calls, or to understand how the neural firing is timed within the structure, HVC is important not only for birdsong in this species. It is also important for the production of at least some calls, including the *gargle* and *chick-a-dee* calls.

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Chapter 4

4 The effects of song and calls on the auditory telencephalon of black-capped chickadees

4.1 Introduction

Songbirds possess a system of interconnected brain regions that function in the perception of auditory stimuli. (Brenowitz, Margoliash, & Nordeen, 1997; Margoliash, 1997; Vates, Broome, Mello, & Nottebohm, 1996). The ascending auditory pathway is similar to that of mammals. Auditory information travels from the nucleus ovoidalis (OV) to Field L and continues to the caudomedial nidopallium (NCM) and the caudomedial and caudolateral sections of the mesopallium (CMM and CLM respectively). CMM and NCM perform functions similar to those of the secondary auditory cortex in mammals (Jarvis et al., 2005; Mello, Velho, & Pinaud, 2004; Pinaud & Terleph, 2008). Electrophysiological studies have shown that these auditory regions are more responsive to the playback of conspecific vocalizations compared to heterospecific vocalizations, pure-tones and white-noise (Grace, Amin, Singh, & Theunissen, 2002; Stripling, Volman, & Clayton, 1997; Theunissen et al., 2004). Thus the auditory forebrain is particularly tuned to vocalizations from birds of the same species and their vocalizations, compared to other species of bird.

In addition to electrophysiological recording, another way to examine activity within the brain is by measuring the expression of immediate-early genes such as ZENK (an acronym for a gene previously known as zif-268, egr-1, NGFI-A and krox-24) and its protein. Zebra finches and canaries both show increased labeling of ZENK mRNA in CMM and NCM following playback of conspecific vocalizations, compared to

heterospecific vocalizations, pure tones, or silence (Mello & Clayton, 1994; Mello, Vicario, & Clayton, 1992). A variety of other bird species also show increased ZENK response in auditory forebrain regions in response to playback of vocalizations: starlings (*Sturnus vulgaris*) (Duffy, Bentley, & Ball, 1999; Farrell, Neuert, Cui, & MacDougall-Shackleton, 2015; Gentner, Hulse, Duffy, & Ball, 2001; Heimovics & Riters, 2007), hummingbirds (*Aphantochroa cirrhochloris*) (Jarvis et al., 2000), house finches (*Carpodacus hirsuta*) (Hernandez & Macdougall-Shackleton, 2003), and, most importantly for this study, black-capped chickadees (*Poecile atricapillus*) (Avey, Kanyo, Irwin, & Sturdy, 2008; Hahn et al., 2015; Phillmore, Bloomfield, & Weisman, 2003; Phillmore, Veysey, & Roach, 2011; Roach, Lockyer, Yousef, Mennill, & Phillmore, 2016). These studies suggest that, across bird species, auditory forebrain regions including CMM and NCM are likely candidates for the processing of higher order auditory information such as call type.

A variety of factors have been shown to influence activity within the brain, specifically in the auditory forebrain. Chickadees are of particular interest because they produce a wide variety of learned vocalizations. Black-capped chickadees not only learn and produce their *fee-bee* song (Kroodsma, Albano, Houlihan, & Wells, 1995; Shackleton & Ratcliffe, 1993), but also produce a variety of other calls that vary in complexity (defined as a vocalization with more notes, more rapid frequency modulations and larger frequency ranges), including the *gargle* and the *chick-a-dee* calls (for complete repertoire see Ficken, Ficken, & Witkin, 1978). The calls are used to demonstrate aggression, alert others of the presence of a predator and maintain contact with members of a flock, and are therefore crucial for individual chickadees' survival (Otter, 2007).

Factors that have been shown to affect ZENK-immunoreactivity (ZENK-ir) in chickadees are type of vocalizations (i.e., *chick-a-dee* call vs. *fee-bee* song) (Avey et al., 2008), rearing conditions (i.e., raised with or without adults) (Hahn et al., 2015) and breeding condition (Phillmore et al., 2011).

Prior studies comparing the ZENK response of auditory brain regions in chickadees following playback of vocalizations have produced contradictory results. For instance, the *chick-a-dee* call has been shown to induce more (Avey et al., 2008) but also less (Phillmore et al., 2003) ZENK-ir than the *fee-bee* song in the auditory telencephalon. Since songbirds, including black-capped chickadees, produce more *fee-bee* songs in the springtime during mating season, the differences in the results of these experiments are attributed to season. During the breeding season, when the production of the *fee-bee* song is at its peak, then the ZENK response in the auditory regions is greater for the *fee-bee* song than the *chick-a-dee* call (Avey et al., 2008). However, at other times of the year, when *fee-bee* song production is less common, than the ZENK response in the auditory regions is greater for the *chick-a-dee* call than the *fee-bee* song. (Phillmore et al., 2003). However, another plausible explanation is that this difference in ZENK-ir in the auditory forebrain can be attributed to the differences in stimulus complexity. In starlings, females show much more ZENK-ir to longer and more complex songs (Gentner et al., 2001). Therefore, this increase in ZENK-ir in the auditory regions when *chick-a-dee* calls are presented could be due to the fact that the *chick-a-dee* call is more acoustically complex than the simple two note *fee-bee* song.

ZENK-ir in the auditory forebrain reflects two possible processes, neither of which are mutually exclusive. The first is that the ZENK-ir reflects the auditory memory

of song, and is therefore the results of the heard stimulus and the memory of the tutor song (typically the father's song). There is a positive correlation between the IEG expression in a nucleus in the auditory forebrain, NCM, and the number of song elements that a bird has successfully copied from their tutor (Bolhuis, Hetebrij, Den Boer-Visser, De Groot, & Zijlstra, 2001). Therefore, when zebra finches are tutored socially, they show localized IEG expression in response to tutor song exposure, which in turn correlates with the strength of song-learning. In turn, female zebra finches raised with their fathers show preferences for the father's song later in life, which is reflected in more IEG expression in CMM (Terpstra, Bolhuis, Riebel, Van Der Burg, & Den Boer-Visser, 2006). Zebra finches also have increased IEG expression in CMM during the sensory phase of song-learning (Gobes, Zandbergen, & Bolhuis, 2010). They also show more IEG expression for their tutor songs compared to novel zebra finch songs in CMM and NCM (Gobes, Zandbergen, & Bolhuis, 2010). Therefore CMM and NCM may serve as neural substrates for tutor song memory. However, another perspective is that IEG expression in the auditory forebrain is related to attention or acoustic complexity (defined as a vocalization with more notes, more rapid frequency modulations and larger frequency ranges) of the stimulus presented. Zebra finches show a decrease in ZENK-ir after repeated exposure to the same song; however, when exposed to a novel song, ZENK-ir increases in the auditory forebrain (Mello, Nottebohm, & Clayton, 1995). Song-sparrows also show increased ZENK-ir to the presentation of a novel song compared to a familiar one (McKenzie, Hernandez, & MacDougall-Shackleton, 2006). Female European starlings also show increased ZENK-ir in NCM to the presentation of a longer, more complex song, compared to a shorter one (Gentner, Hulse, Duffy, & Ball, 2001).

Therefore, both views are supported in the literature: it is possible that IEG expression in the auditory forebrain could be due to the auditory memory of that vocalization or to the animal paying attention to the stimuli being presented, or the acoustic complexity of the auditory stimuli.

For many songbirds the song they produce is their most complex vocalization and it is also the most salient to the animal, as it often conveys an animal's phenotype to a potential partner. Therefore, the two possible roles of the auditory forebrain structures are somewhat confounded. The black-capped chickadee is the perfect candidate to investigate the neural basis of perception in the auditory forebrain because they produce a simple song, the *fee-bee*, which is learned early in life, and therefore should form a memory template in the auditory forebrain. They also produce calls that are partially learned, but much more acoustically complex like the *gargle* or *chick-a-dee* calls. Therefore, if we observe more IEG expression in CMM and NCM for the *fee-bee* song, this would reflect the auditory memory for that song. However, if we see more IEG expression for the *gargle* or *chick-a-dee* calls, it could be due to the acoustic complexity of the vocalizations.

The *gargle* call has been largely overlooked in studies of the ZENK response to vocalizations in chickadees. It is an extremely acoustically complex call, and is also produced year round, and more so in the summer months (Ficken et al., 1978). Although chickadees have only a single song type (the *fee-bee* song) they do have a *gargle* call repertoire. Most chickadees can have as many as 10 *gargle* call types (Ficken, Weise, & Reinartz, 1987). It seems like the *gargle* call would be a good candidate to study the processing of higher order auditory information in CMM and NCM. Chickadees are

therefore a notable exception compared to most songbirds; their song is less acoustically complex than their *chick-a-dee* and *gargle* calls (Otter, 2007). In contrast to my results in Chapter 2, only one other study has examined this indirectly, where during the playback of *fee-bee* song, and components of the *fee-bee* song, chickadees produced the *gargle* call, and the number of *gargle* calls produced did not correlate with the amount of neural activation observed in HVC (Roach et al., 2016).

The objective of this study was to examine the effect of different calls and song playbacks on ZENK activation in CMM and NCM, and whether this activation is modulated by the complexity of the vocalization, or by the function of the vocalization. I tested this by capturing black-capped chickadees and putting them in social and acoustic isolation from one another before exposing them to recorded playback stimuli (see Figure 4-1). The birds were separated by sex; males and females, and then randomly assigned to different playback conditions (i.e., *fee-bee* song, *gargle* call, *chick-a-dee* call, pink-noise and silence). The birds listened to 30 minutes of vocalizations, and, following the playback, birds were euthanized and the brains collected for processing. I used the immediate-early gene ZENK to quantify the amount of neuronal activation in CMM and NCM during the different playback conditions (Jarvis & Nottebohm, 1997). I predicted that if the activation was modulated by call complexity, I would see the highest amount of ZENK-ir in CMM and NCM for the *gargle* call, followed by the *chick-a-dee* call and then the *fee-bee* song. Whereas if the activation were modulated by the function of these vocalizations, I would predict that the *fee-bee* song (used primarily to attract a mate and defend one's territory) would show the most ZENK-ir, with the *gargle* (an aggressive

vocalization) and *chick-a-dee* calls (a mild alarm or contact/group cohesion call) showing similar but lesser levels of ZENK-ir in CMM and NCM.

4.2 Methods

4.2.1 Subjects and handling

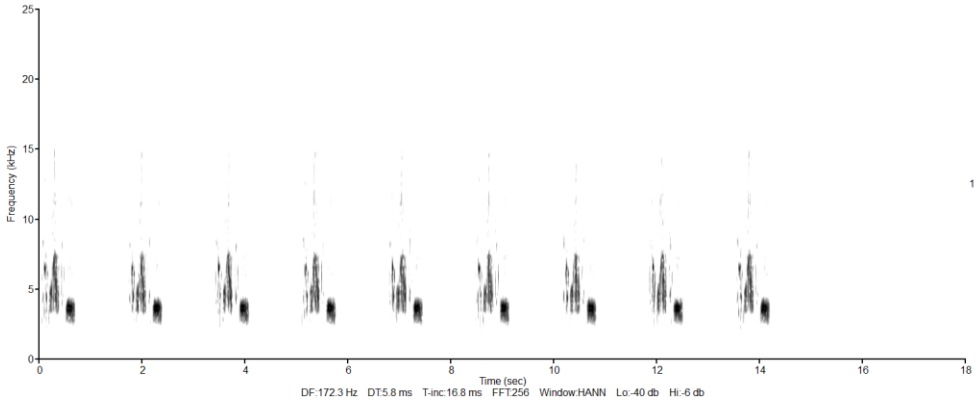
During the winter season from September 2014 to January 2016, I captured 33 adult black-capped chickadees (*Poecile atricapillus*) on the campus of the University of Western Ontario, London, Ontario (43˚01’ N, 81˚27’ W). I identified birds as either male (*n* =15) or female (*n* =18) based on body mass and wing chord measurements, which I later confirmed by examining the gonads post-mortem. Birds were initially group-housed (range: 3-4 birds per cage) in an outdoor aviary. Birds had ad libitum access to food (Mazuri small-bird maintenance diet mixed with black-oil sunflower seeds) and water; their diet was also supplemented with mealworms (2 worms per individual per day). Following quarantine, I moved individual birds into social and acoustic isolation in a wire cage (25 cm × 30 cm × 37 cm) lined with newspaper placed inside modified audiometric testing booth (width 91cm X height 172cm X depth 71cm, Industrial Acoustics Company, Inc., Bronx, NY). The birds continued to have ad-libitum access to food and water. The photoperiod inside the isolation chamber was set to match the outdoor ambient daylight cycle. The birds remained in isolation for a period of at least 24 hours before they were exposed to vocal playbacks.

4.2.2 Playback procedure

4.2.2.1 Playback stimuli

Using RavenPro 1.4 (Bioacoustics Research Program, 2011), I used recordings obtained in previous studies (see Chapter 2), as well as samples found on the Cornell Lab of Ornithology website (https://www.allaboutbirds.org/) to construct four different kinds of audio stimuli: (1) *fee-bee* song, (2) *gargle* call, (3) *chick-a-dee* call, and (4) pink-noise. Each group had three different stimulus sets consisting of four vocalizations produced by three black-capped chickadees, where no calls were repeated between stimulus sets (i.e., $A_1B_1C_1A_2$, $B_2C_2A_3B_3$ and $C_3A_4B_4C_4$; where the letter represents the bird producing the vocalization, and the number represents the particular vocalization). Vocalizations were bandpass-filtered between 1000 and 22,000 Hz using RavenPro 1.4 (Bioacoustics Research Program, 2011) to remove background noise, and the amplitude was equalized across vocalizations. Each individual vocalization was repeated for a period of 15-s with 1-s intervals between them, followed by 45-s of silence (See Figure 4-1), to form a 60-s sequence (following Avey et al., 2011). This 60-s sequence was repeated 30 times to make a 30-min playback stimulus. For the pink-noise stimuli, I constructed three different stimuli; each one matched to the mean duration of each of the three other vocalization types, and cropped white noise stimulus within the average frequency ranges for each vocalization used in the study (i.e., *fee-bee* song, *gargle* call and *chick-a-dee* call). All other parameters remained the same. The total amount of vocalizing in the 30-min playback was also controlled for across groups, differing in at most 2-s total across different playback conditions. There was also a silent control condition where no auditory playback was presented at all.

A)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Time (sec)
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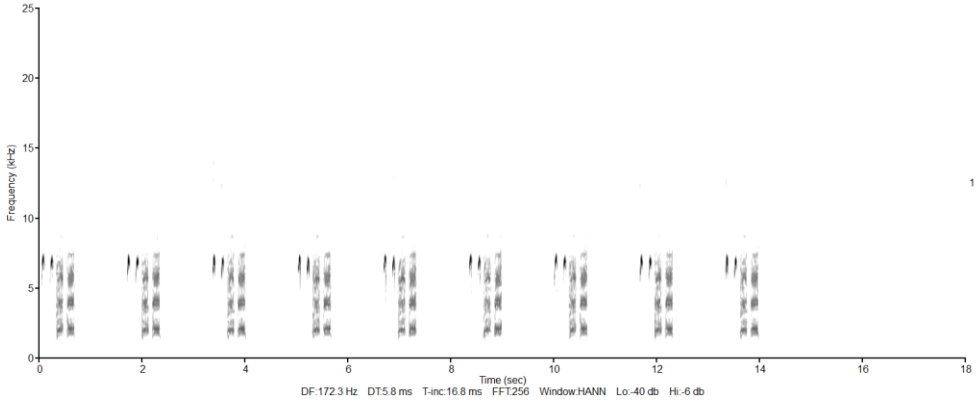
B)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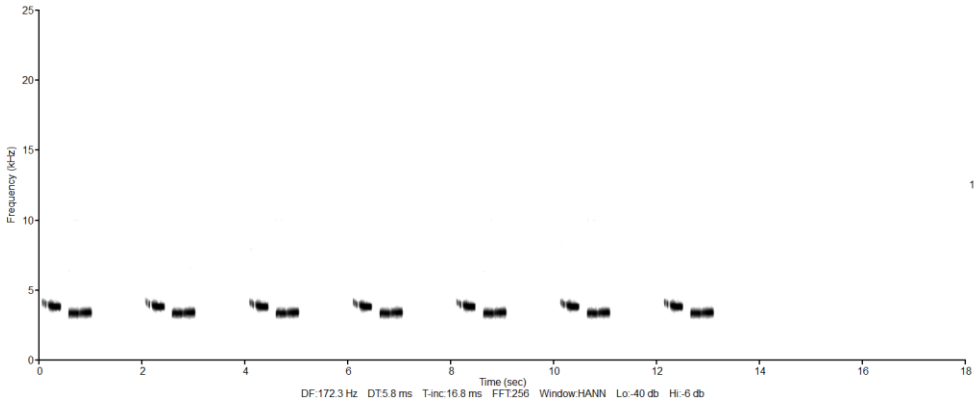
C)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D)

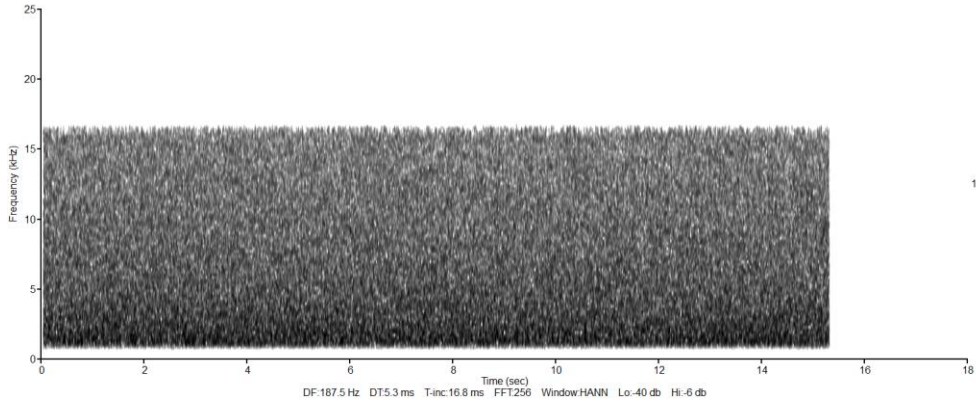


Figure 4- 1 Examples of the different vocalizations for the different playback groups. Each spectrogram represents the time in seconds on the x-axis and the frequency in kHz on the y-axis. Each vocalization is played followed by a 1-s period of silence before the next vocalization. This is repeated until the sample is approximately 15 s in length, and then followed by a 45 s period of silence and then repeated. A) Sample *gargle* call playback vocalizations, B) sample *chick-a-dee* call playback vocalizations, C) sample *fee-bee* song playback vocalizations, and D) pink-noise playback stimulus.

4.2.2.2 Playback equipment and procedure

Between June and July 2016, I randomly assigned chickadees to each of the five playback conditions (silence, *gargle* calls, *chick-a-dee* calls, *fee-bee* song, or pink-noise) while ensuring balanced sex ratios. I moved the birds into individual cages (25 cm × 30 cm × 37cm) inside a modified audiometric testing booth (width 91cm X height 172cm X depth 71cm, Industrial Acoustics Company, Inc., Bronx, NY) 24 h prior to the playback. The photoperiod inside the isolation chamber was set to match the outdoor ambient daylight cycle, and the birds had ad libitum access to food and water. Prior to moving the individual bird into isolation, I outfitted each audiometric testing booth with one pair of speakers (Koss HDM/111BK) attached to a HipStreet (model HS-636-4GBBL) mp3 player located outside of the chamber, preventing the bird from being disrupted when I began the playback treatments. I also installed a webcam (Logitech HD pro webcam

C920) to the ceiling of the chamber attached to a USB port outside the chamber to allow recording and verify that the bird was not vocalizing during the playback. Prior to the playback, the lights in the chamber were turned off for 1 h, then the playback was started for 30 min, and the bird then remained in silence and dark chamber for an additional 1 h. For birds in all of the above groups, following the hour of isolation, I anesthetized birds using isoflurane. Following deep anesthesia, birds were euthanized by transcardial perfusion with 0.1M phosphate buffered saline (PBS) followed by buffered 4% paraformaldehyde. I quickly removed the brain from the skull and placed it in 4% paraformaldehyde (~24 h) and then in 30% sucrose (~36 h) at 4 ˚C. Brains were frozen on crushed dry ice and then stored at -80 ˚C.

4.2.3 ZENK immunohistochemistry

I ran immunohistochemistry in multiple runs counterbalanced across the different playback groups. I used an established ZENK immunohistochemistry protocol where multiple sections were contained in wells in tissue-culture trays, and the solutions were pipetted in and out of each individual well (Farrell, Neuert, Cui, & MacDougall-Shackleton, 2015; Hernandez & MacDougall-Shackleton, 2004; Maney, MacDougall-Shackleton, MacDougall-Shackleton, Ball, & Hahn, 2003; McKenzie, Hernandez, & MacDougall-Shackleton, 2006; Schmidt, McCallum, MacDougall-Shackleton, & MacDougall-Shackleton, 2013). Using a cryostat, I sectioned brains along the sagittal plane in 40 µm sections and temporarily stored them in 0.1M PBS. Every second section (i.e., 80 µm interval) was used to examine ZENK immunoreactivity (ZENK-ir). First, free-floating sections were thoroughly rinsed twice with 0.1M PBS, and then incubated with 0.5% H_2O_2 in PBS for 15-min to eliminate endogenous peroxidase activity. Sections

were washed three times with 0.1 M PBS, and then incubated in 10% Normal Goat Serum (cat no. S-1000; Vector Laboratories, Burlingame, CA USA) in 0.1 M PBS containing 0.3% Triton X-100 (0.3% PBS/T) for 1 h. Sections were then incubated with primary antibody made in rabbit against Egr-1 (polyclonal, 1:4000, cat no. SC-189; Santa Cruz Biotechnology, Santa Cruz, CA USA) in 0.3% PBS/T for ~24 h at 4 ˚C. After rinsing three times with 0.1% PBS/T, sections were incubated with biotinylated goat anti-rabbit IgG secondary antibody (1:250 dilution) for 1 h at room temperature, followed by three rinses with 0.1% PBS/T. Sections were then incubated with avidin-biotin horseradish-peroxidase complex (VectaStain Elite ABC Kit, cat no. PK 6100; Vector Laboratories) at dilution 1:200 for 1 h, followed by two rinses with 0.1% PBS/T. The tissue sections' immunoreactivity was then visualized with 3, 3'-diaminobenzidine tetrahydrochloride (SigmaFAST DAB, cat no. D4418; Sigma). After thoroughly rinsing the sections with PBS, I mounted the sections onto electrostatically treated microscope slides (VWR VistaVision™ Histobond ®) and left to dry overnight. Once dry, I put the slides through serial dehydrations with increasing concentrations of ethanol, and cleared of lipids with an organic solvent (NeoClear, cat no. 65038-71; EMD Chemicals, Mississauga, Ontario, Canada). Finally slides were covered with coverslips using a mounting medium (Permount, cat no. SP15; Fisher Scientific) and allowed to dry in a fume hood ~12 h.

4.2.4 ZENK quantification

ZENK-ir was quantified for three auditory regions: CMM, dorsal NCM (NCMd) and ventral NCM (NCMv, see Figure 4-2) by using a Leica DM 5500B microscope coupled to a Leica 420C camera. For each chickadee, 10 to 12 images were captured for

CMM (~5-6 images/hemisphere), NCMd (~5-6 images/hemisphere), and NCMv (~5-6 images/hemisphere). I began quantifying ZENK expression on the first, most medial, section in which the mesopallium was contiguous with the rostral portion of the nidopallium to make sure that the orientation of the nidopallium was correct. The sections were selected such that the image was contained completely within the structure. For NCMd the images were taken from the most dorso-caudal part of NCM, and for NCMv images were obtained from the most ventro-rostral part of NCM (see Figure 4-2). CMM

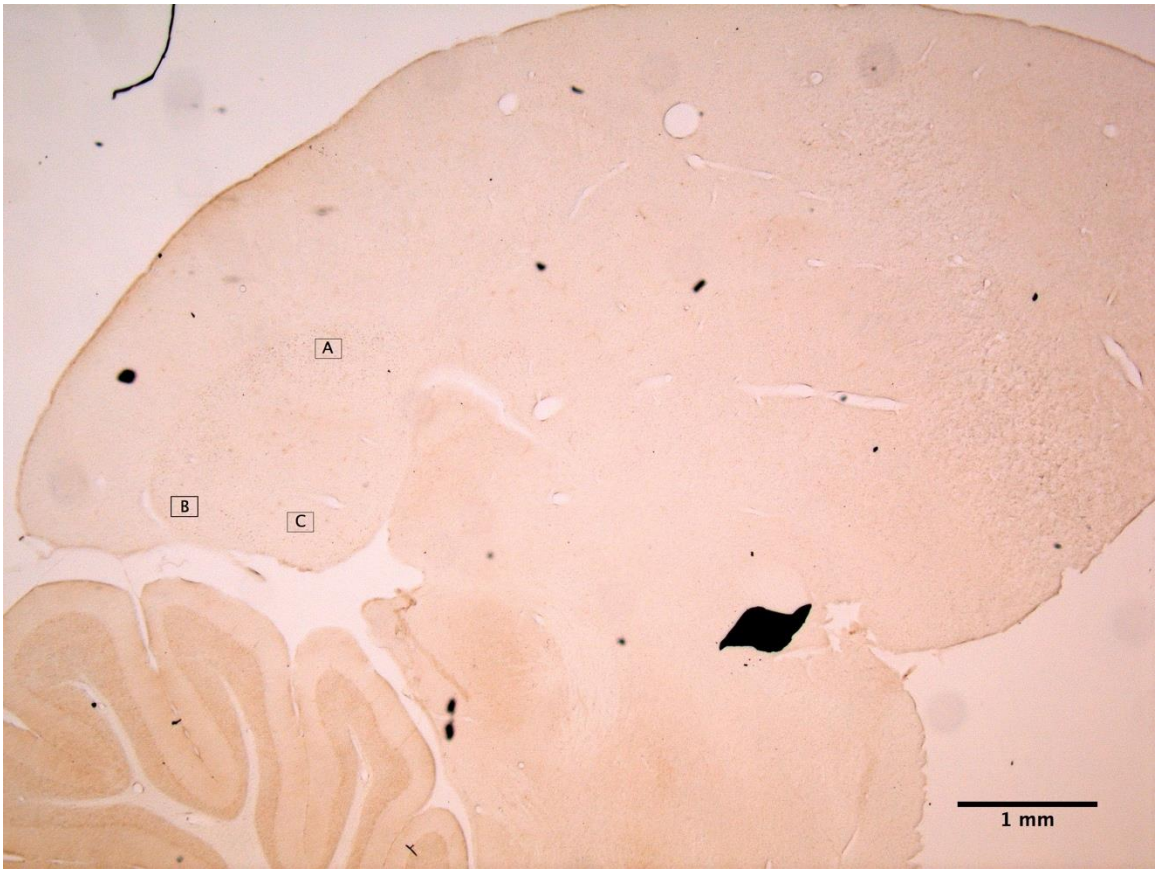


Figure 4- 2 Sagittal slice of black-capped chickadee auditory forebrain. Sampling region used to quantify ZENK-ir in CMM (A), NCMd (B) and NCMv (C). Left is dorsal and right is caudal. The boxes are not representative of the actual scale of the sampling area, but are to demonstrate the location where the images were taken.

images were acquired from the most caudal part of the structure, and in all regions the images were taken from the area of highest immune-positive ZENK cells within the area (following Gentner et al., 2001; Hernandez & MacDougall-Shackleton, 2004 ; Avey, Phillmore, & MacDougal-Shackleton, 2005; Schmidt, McCallum, MacDougall-Shackleton, & MacDougall-Shackleton, 2013). For each field of interest, z-stack images of 0.63 µm steps through the focal planes were collected through the 20× objective lens and were then compiled using a montage mode in Leica Application Suite software, the observer was blind to the sex and experimental condition of the bird. This allowed for all of the ZENK-ir cells to be in focus within the same image. For each image, the area (mm^2) was determined by using a calibration image also taken with the 20× objective lens. I counted the number of ZENK-ir cells following a semi-automated protocol using the ImageJ program (NIH). Briefly, images were opened in ImageJ and were automatically adjusted to gray scale, autocontrasted and auto-thresholded. The threshold was adjusted in order to ensure that only immunoreactive cells were highlighted. Minimum and maximum cell sizes were based on prior studies were used to exclude non-cell objects (9.07-27.21 µm) and a minimum sphericity of 0.65 was used in ImageJ during the cell counting procedures. The measurements for area (mm^2) and cell counts were entered in a spreadsheet and the number of cells/mm^2 was determined in order to control for any size differences in CMM and NCM across individual birds.

4.2.5 Data and statistical analyses

Statistical analyses were carried out using IBM SPSS Statistics 24.0. The mean number of cells/mm^2 for each individual was compared among the right and left hemispheres using a paired t-test. No significant differences were found between

hemispheres; therefore all analyses were conducted on the mean cell count per mm^2 pooled across hemispheres.

A 3-way repeated measures ANOVA was run to determine the effect of the different playback conditions on the number of ZENK-ir cells in HVC and RA, with brain region (CMM, NCMd, NCMv) as a within-subjects factor, different playback conditions (*fee-bee*, *chick-a-dee*, *gargle*, pink noise, and silence) as a between-subjects factor, and sex (male and female) as a between-subjects factor. The dependent variables were the ZENK-ir (cells/mm^2) in CMM, NCMd and NCMv respectively. Results were considered significant at $\alpha \leq 0.05$ level. Data are presented as mean ± SEM.

4.3 Results

The initial ANOVA revealed that there was a significant main effect of brain region, $p < 0.05$, but no interactions, $p > 0.05$. Therefore three separate 2-way ANOVAs were run for each of the three auditory brain regions; CMM, NCMd, and NCMv. The between-subject factors were sex (male and female) and playback condition (*chick-a-dee*, *gargle* calls, *fee-bee* song, pink-noise and silent controls), the dependent variables were the ZENK-ir (cells/mm^2) in CMM, NCMd and NCMv respectively. Results were considered significant at $\alpha \leq 0.05$ level. Data are presented as mean ± SEM.

4.3.1 CMM

There was no significant interaction between sex and playback conditions on ZENK-ir in CMM, $F(4,22) = 0.335$, $p = 0.851$. A main effect of playback condition was obtained, $F(4, 22) = 5.11$, $p = 0.005$. The birds in the *gargle* call playback condition

showed significantly more activation in CMM than those in the pink-noise condition, p = 0.006, and the silent control condition, p = 0.011 (see Figures 4-3 & 4-4). No other

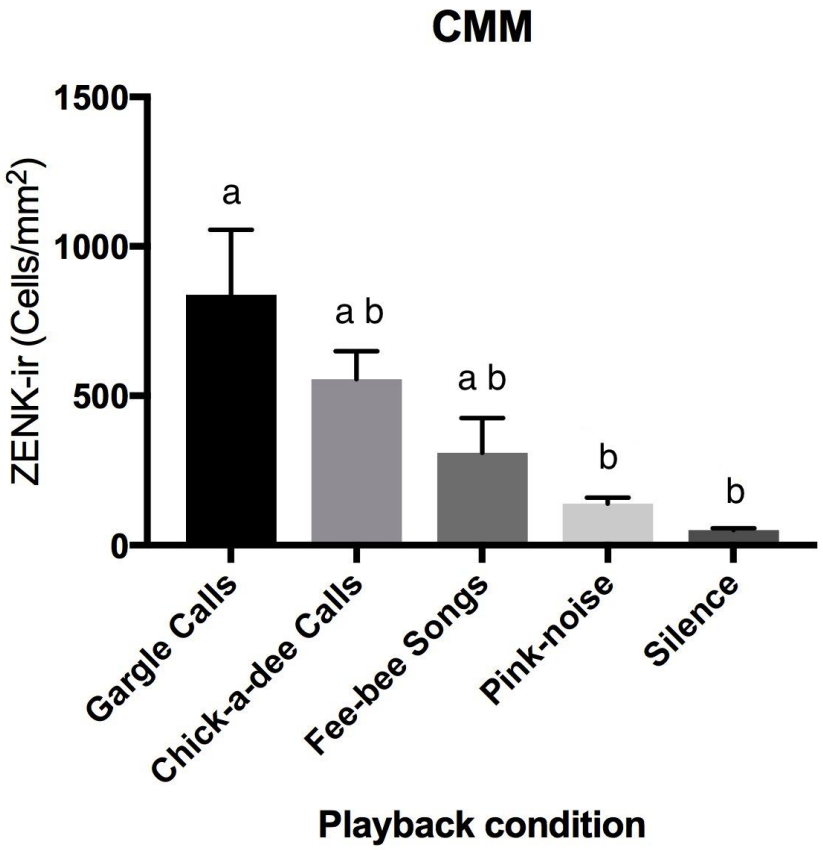


Figure 4- 3 Effect of playback vocalization on the total number of ZENK-ir cells in CMM of adult black-capped chickadees. The birds in the *gargle* playback group had more ZENK-ir cells in CMM than the pink-noise and silent control groups. The letters represent statistical differences between the groups; letters that share the same lower case letter did not significantly differ from each other.

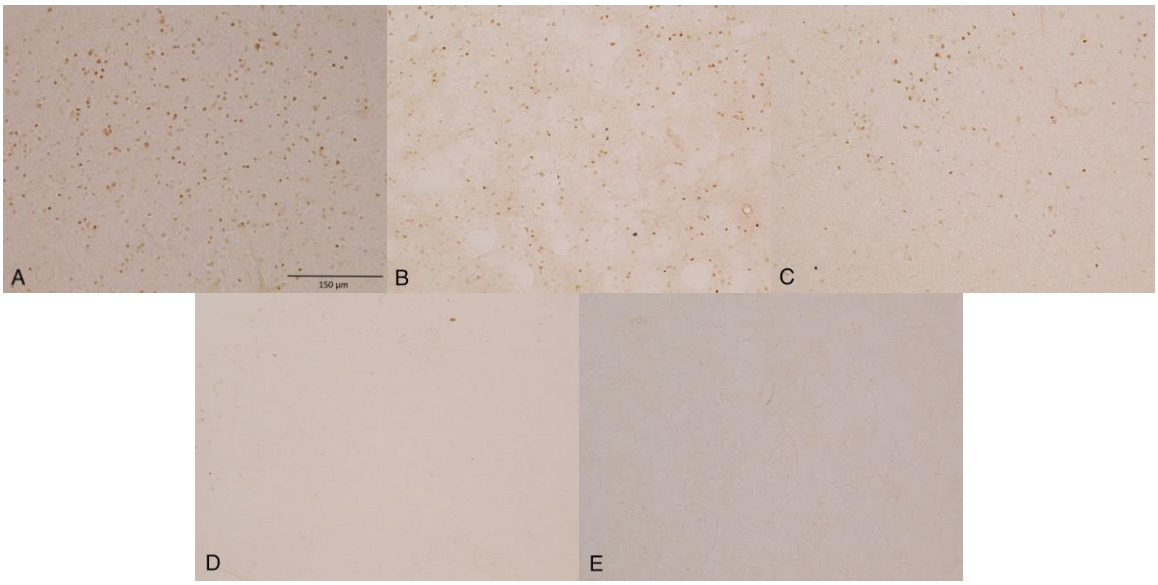


Figure 4- 4 Example ZENK-ir in CMM of black-capped chickadees to each of the five playback conditions. The ZENK-ir is visible for the *gargle* (A), *chick-a-dee* (B), *fee-bee* (C), and pink-noise playbacks (D). All images were taken at the same magnification. Silent controls are also shown (E).

playback condition differed from any other in ZENK-ir in CMM, $p > 0.05$. No main effect of sex was obtained, $F(1,22) = 0.292$, $p = 0.594$.

4.3.2 NCMd

There was no significant interaction between sex and playback condition on ZENK-ir in NCMd, $F(4,22) = 0.330$, $p = 0.855$. A main effect of playback condition was obtained, $F(4, 22) = 3.938$, $p = 0.015$. The birds in the *gargle* call playback condition showed significantly more activation in NCMd than those in the silent control condition, $p = 0.033$ (see Figures 4-5 & 4-6). No other playback condition differed from any other in ZENK-ir in CMM, $p > 0.05$. No main effect of sex was obtained, $F(1,22) = 0.483$, $p = 0.494$.

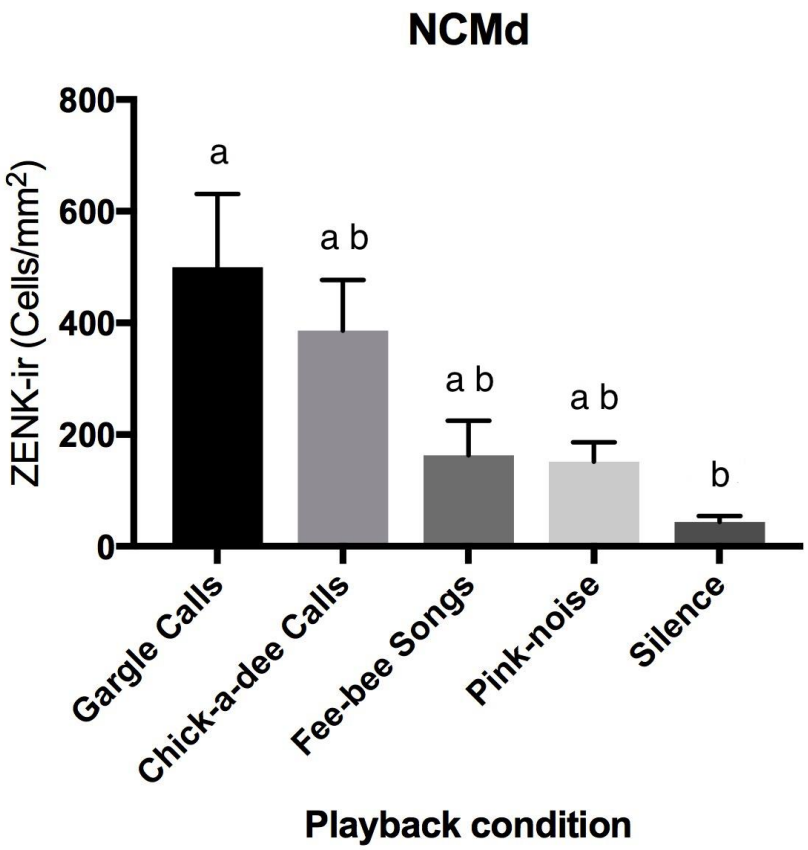


Figure 4- 5 Effect of playback vocalization on the total number of ZENK-ir cells in NCMd of adult black-capped chickadees. The birds in the *gargle* playback group had more ZENK-ir cells in NCMd than the silent control group. The letters represent statistical differences between the groups; letters that share the same lower case letter did not significantly differ from each other.

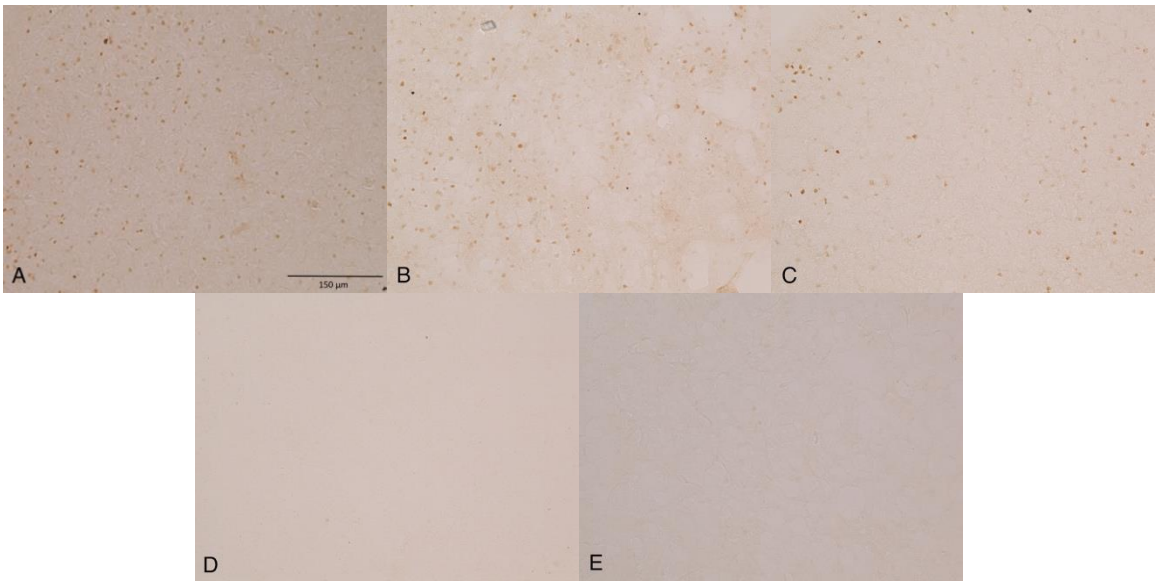


Figure 4- 6 Example ZENK-ir in NCMd of black-capped chickadees to each of the five playback conditions. The ZENK-ir is visible for the *gargle* (A), *chick-a-dee* (B), *fee-bee* (C), and pink-noise playbacks (D). All images were taken at the same magnification. Silent controls are also shown (E).

4.3.3 NCMv

There was no significant interaction between sex and playback conditions on ZENK-ir in NCMv, $F(4,22) = 0.085$, $p = 0.986$. A main effect of playback condition was obtained, $F(4, 22) = 3.188$, $p = 0.033$. None of the playback conditions were significantly different from one another, $p > 0.05$, however some were approaching significance (see Figures 4-7 & 4-8). The birds in the *gargle* call playback condition had almost significantly more activation in NCMv than the pink-noise, $p = 0.062$, and the silent control groups, $p = 0.070$. No main effect of sex was obtained, $F(1,22) = 0.141$, $p = 0.711$.

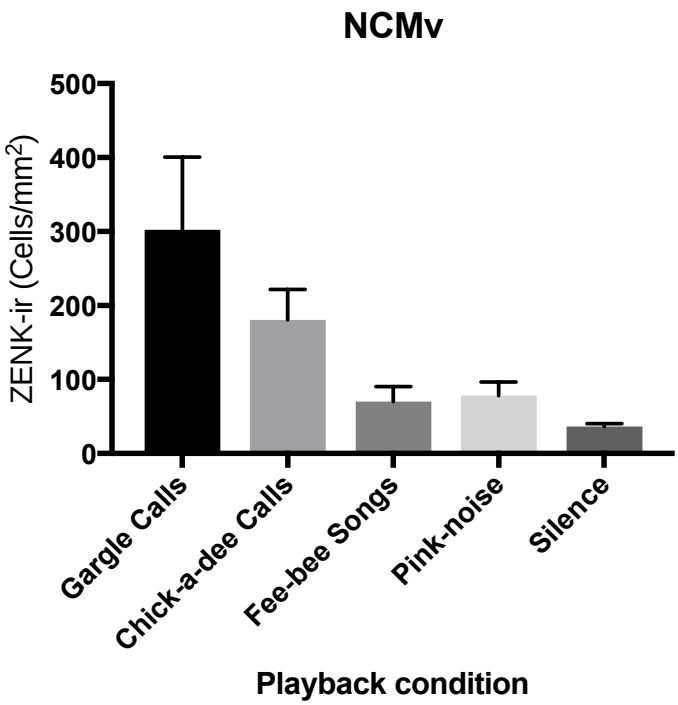


Figure 4- 7 Effect of playback vocalization on the total number of ZENK-ir cells in NCMv of adult black-capped chickadees. No vocalization condition was significantly different from any other.

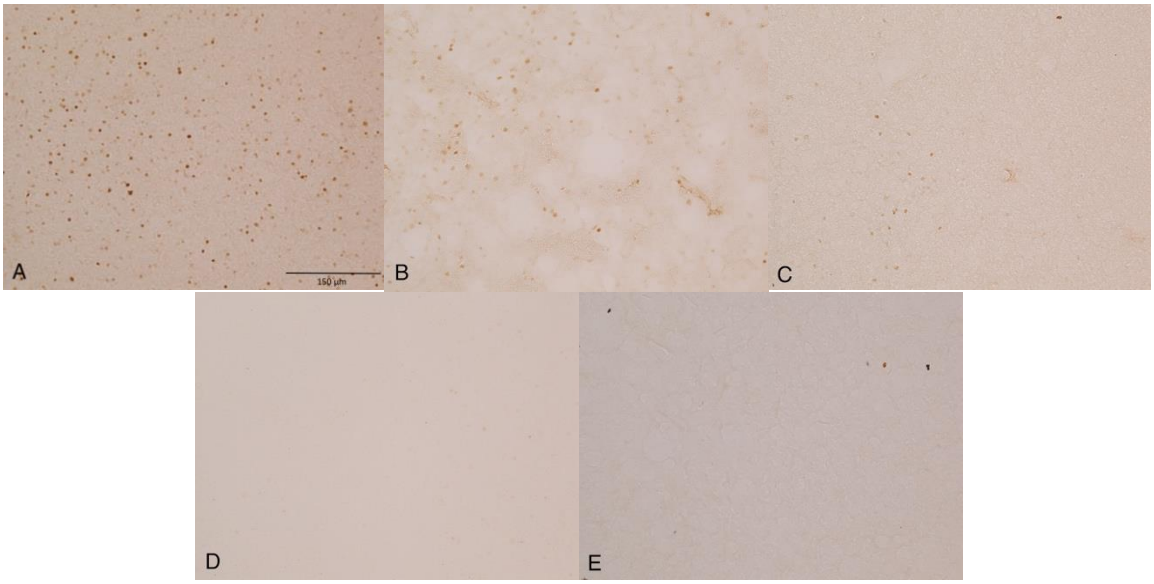


Figure 4- 8 Example ZENK-ir in NCMv of black-capped chickadees to each of the five playback conditions. The ZENK-ir is visible for the *gargle* (A), *chick-a-dee* (B), *fee-bee* (C), and pink-noise playbacks (D). Silent controls are also shown (E).

4.4 Discussion

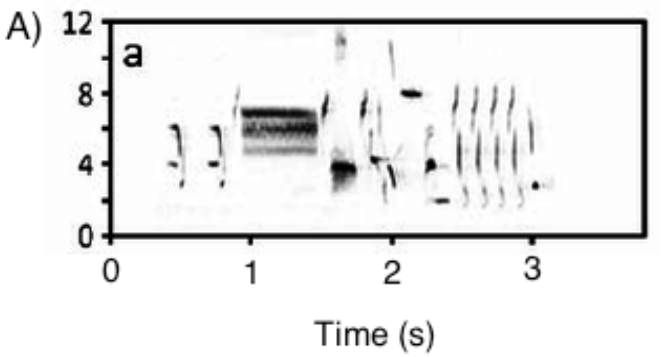
This study was conducted to determine if there was a difference in neural activation (ZENK-ir) to the playback of different black-capped chickadee vocalizations in CMM and NCM and if these differences in ZENK-ir would be due to (a) acoustic complexity, or (b) the function of the vocalizations. The data support the conclusions that there are differences in the amount of ZENK-ir in the auditory regions when presented with the different vocalizations of the black-capped chickadee, and that these differences could be due to the acoustic complexity of the vocalizations, and not the function of the vocalization.

4.4.1 CMM and NCMd

The playback of the *gargle* call vocalization elicited the most ZENK-ir in CMM. This indicates that the most neurons within CMM were repeatedly depolarized when listening to the *gargle* vocalization playback compared to the other playback conditions. The playback of the *gargle* vocalizations was the only one to elicit significantly different ZENK-ir compared to pink-noise and silent controls. However, the number of ZENK-ir cells did not differ between birds listening to the *gargle* calls, *chick-a-dee* calls or *fee-bee* songs. Although not statistically significant, there was a trend observed where the *gargle* call playbacks elicited the most neural activation, followed by the *chick-a-dee* calls, the *fee-bee* songs, pink-noise and finally silent controls. Also there were no differences in the amount of ZENK-ir cells in CMM between males and females. In NCMd the same trend was observed, except that there were no significant differences in the amount of ZENK-ir cells between the birds who listened to the *gargle* call and those who listened to the pink-noise. Similarly, there were no differences in ZENK-ir cells between the birds who

listened to the *chick-a-dee* calls, the *fee-bee* songs, pink-noise and silent controls. Although non-significant, I also observed the same trend where the most neuronal activation is observed for birds who listened to the *gargle* calls, followed by those who listened to the *chick-a-dee* calls and then the *fee-bee* songs.

These results are similar to those found by Avey and colleagues (2008), where the playback of the *chick-a-dee* call induced the most ZENK-ir in CMM compared to the *fee-bee* song, whereas in NCMd the amount of ZENK-ir did not differ between the *chick-a-dee* call playback and the *fee-bee* song playback. However, unlike that study, I did not find a difference between males and females. In their study they used both male and female *chick-a-dee* calls and *fee-bee* songs, and suggested that the particular minute differences in acoustic features between male and female calls are influencing the amount of ZENK-ir (Avey et al., 2008). This does not seem to be the case for the current study. It is possible that CMM and NCMd are tuned to the complexity of the acoustic stimulus presented, where the more complex a vocalization, the more ZENK-ir response is observed. Chickadees in non-breeding condition, as they would have been in this study, show greater ZENk-ir cells in CMM and NCMd when listening to a heterospecific song-sparrow song (see Figure 4-9) playbacks (Phillmore et al., 2011). The song-sparrow song



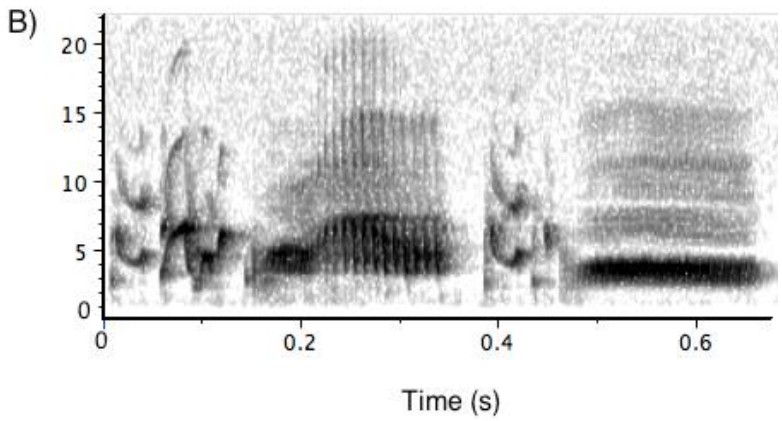


Figure 4- 9 Image adapted with permission from Lapierre, Mennill, & MacDougall-Shackleton (2011) (A). Spectrogram of a song produced by a sparrow (*Melospiza melodia*), where the y-axis shows the frequency in kHz (A). A spectrogram of a black-capped chickadee (*Poecile atricapillus) gargle* call, where the y-axis shows the frequency in kHz.

is a complex vocalization, with notes varying in frequency. It is composed of a great variety of notes, similar to a *gargle* call (Ficken & Popp, 1992). Therefore it seems likely that the differences in ZENK-ir in CMM and NCMd could be driven by the acoustic complexity of the vocalization not the function of the vocalization, because a song-sparrow song would have little relevance to the black-capped chickadee. Time of year also cannot account for these results. The playbacks were conducted during the months of June and July, which is a time when no vocalization that the chickadee produces is at its peak (Avey, Quince, & Sturdy, 2008), therefore there are no biases where the birds would be particularly tuned to one of their vocalization in their environment. These results support the idea that IEG expression in the auditory forebrain is due to acoustic complexity of the vocalization and not due to a memory template for the vocalization. Songbirds may be sensitive to the acoustic features of vocalizations in the auditory forebrain, and that more acoustically complex vocalizations induce more neural firing within the auditory forebrain.

4.4.2 NCMv

NCMv had much less ZENK-ir than the two other auditory regions; CMM and NCMd. There was also no difference in the ZENK-ir induction between the different stimulus types: *gargle* calls, *chick-a-dee* calls, *fee-bee* songs, pink-noise and silence. This decrease in the amount of neuronal activation as well as the lack of differences in NCMv is consistent with a trend that is observed when moving down the ascending auditory pathway and is in accordance with previous songbird ZENK-ir studies (Avey et al., 2008; Phillmore et al., 2003).

4.4.3 Conclusions

It is not surprising that the perception of calls and song in black-capped chickadees seems to be modulated by call complexity, as a similar phenomenon is observed in European starlings (Gentner et al., 2001). In this case females showed more ZENK-ir in NCM to more complex songs than to simpler songs. Just like most oscine birds, black-capped chickadees learn their song, the *fee-bee* (Shackleton & Ratcliffe, 1993), but they also partially learn the majority of their calls (Baker, Howard, & Sweet, 2000; Guillete et al, 2011; Hughes, Nowicki, & Lohr, 1998), which could mean that their auditory regions could be sensitive not only to song, but to other vocalizations. It also suggests that because these calls are only partially learned, that the auditory forebrain is tuned to the acoustic features of the vocalizations, therefore we observe more repeated depolarization in these regions for more acoustically complex vocalizations. And unlike the results obtained in zebra finches, where NCM seems to be part of the neural substrates for storage of song memory, black-capped chickadees do not show the most

ZENK-ir for the *fee-bee* song indicating that CMM and NCM may play a role in the perception of the complexities of all vocalizations (Bolhuis & Gahr, 2006).

Alternatively, the salience of the vocalizations presented may play a role in the neural representation of these vocalizations in the auditory regions. The *gargle* call is most often followed by an attack from the emitting black-capped chickadee (Ficken et al., 1978). Therefore a chickadee hearing the *gargle* call may need to prepare themselves for an imminent attack and choose whether to fight or flee. Therefore this vocalization may be more salient to the chickadee and induce more neural expression in those secondary auditory areas. It would be worth investigating if predator vocalizations and *gargle* calls, if we control for total amount of vocalizing, would elicit similar levels of ZENK-ir in NCM and CMM since they would have similar salience to the listener.

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Chapter 5

5 Auditory cortex activity in response to female and male long-calls in HVC lesioned male zebra finches

5.1 Introduction

Zebra finches are heavily studied in avian neurobiology because the males learn and memorize their song from a tutor bird, and this learning and memory process is similar to how human infants develop speech (Doupe & Kuhl, 1999, Funabiki & Konishi, 2003; Konishi, 1985). In addition to song, zebra finches also produce a "long-call" or "distance call" which is used in situations when birds are separated from one another visually, but can still hear each other acoustically (Elie & Theunissen, 2016; Zann, 1996). This call is sexually dimorphic, where the male long-call tends to be shorter, has a higher fundamental frequency, is more consistent in their length, and possesses fast frequency modulation which resembles song syllables (Price, 1979; Simpson & Vicario, 1990; Zann, 1984)(see Figure 5-1). Although both males and females use their long-calls

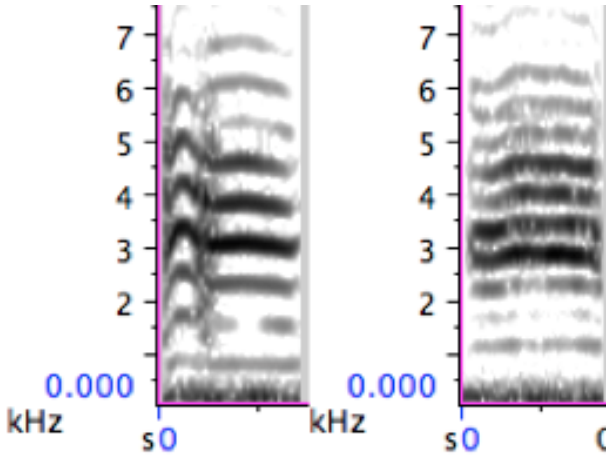


Figure 5- 1 Examples of the male and female long-calls. Each spectrogram represents the time in seconds on the x-axis and the frequency in kHz on the y-axis. The male long-call is on the left and the female long-call on the right. Notice the frequency modulation at the beginning of the male long-call.

in similar contexts, males must learn this call from the same tutor from which they learn their song, whereas for females long-calls are innate (Simpson & Vicario, 1990; Zann, 1985). Zebra finches also respond more to the long-call of their mate than to long-calls from other zebra finches, and it also seems as though they are able to discriminate between male and female calls, and their mate's call from those of another zebra finch (Vicario, Naqvi, & Raksin, 2001; Vignal, Mathevon, & Mottin, 2004, 2008). Thus long-calls share many properties with song, including imitative vocal learning and individual recognition.

The song-control system is a set of discrete brain nuclei that are involved in the learning and production of song (Nottebohm, 2005; Nottebohm & Arnold, 1976). The song-control system seems to be crucial for the perception and production of learned calls as well (Simpson & Vicario, 1990; Ter Maat, Trost, Sagunsky, Seltmann, & Gahr, 2014; Vicario et al., 2001). HVC and the robust nucleus of the arcopallium (RA) are crucial for the production of the male-typical features of the long-call in zebra finches (Simpson & Vicario, 1990). Bilateral HVC lesions caused changes in the fundamental frequency and the fast frequency modulations, and the temporal structure of male long-calls, rendering them more female-like. The same effects were observed following bilateral RA lesions. However, these lesions did not affect the female long-call, demonstrating the importance of HVC and RA in the production of the learned features of the male long-call in zebra finches. HVC also shares a reciprocal connection with a subsection of CMM called nucleus avalanche (Lewandowski & Schmidt, 2011). Therefore it is possible that HVC could modulate sensory input that is reaching the auditory region CMM, and nucleus

avalanche specifically. This is what I investigated in this chapter, the role that HVC plays in the perception of calls in the zebra finch.

Female and male zebra finches seem to prefer the female compared to the male long-call (Vicario, Naqvi, & Raksin, 2001). Males and females both tend to respond, or call back, more to female rather than male long-calls. Auditory forebrain regions, in particular the caudomedial nidopallium (NCM) and the caudomedial mesopallium (CMM), are involved in the perception of songs and calls, and may be regions that contain the memories for calls and songs (Bolhuis, Hetebrij, Den Boer-Visser, De Groot, & Zijlstra, 2001; Bolhuis, Zijlstra, den Boer-Visser, & Van Der Zee, 2000; Bolhuis, Gobes, Terpstra, den Boer-Visser, & Zandbergen, 2012; Chew, Mello, Nottebohm, Jarvis, & Vicario, 1995; Chew, Vicario, & Nottebohm, 1996; Gobes et al., 2009; Mello & Clayton, 1994; Terpstra, Bolhuis, & Den Boer-Visser, 2004; Terpstra, Bolhuis, Den Boer-Visser, & Cate, 2005; Vignal, Andru, & Mathevon, 2005). It seems likely that both the song-control system, as well as parts of the auditory forebrain, are crucial for long-call production and perception. The neuronal response to sexually dimorphic long-calls does not match the behavioural preferences for female long-calls in zebra finches (Gobes et al., 2009). When presented with female long-calls, females showed increased numbers of neurons expressing the immediate-early gene ZENK in CMM and NCM, compared to females who heard silence. However males did not show this pattern, even though they do preferentially respond behaviourally to female rather than male long-calls (Gobes et al., 2009). Recent evidence has shown that female zebra finches presented with female or male long-call show equivalent amounts of number of neurons expression the immediate-early gene ZENK in NCM and CMM (Scully, Hahn, Campbell, McMillan, Congdon, &

Sturdy, 2017). These same findings were also true for males. Therefore it is unclear if female zebra finches show a neural basis of perception difference of female and male long-calls.

There have been very few studies that have investigated the neural basis of perception of call processing, and to my knowledge none that have investigated the contribution of the song-control system to the neural processes underlying call perception. Lesioning RA in male zebra finches reduces their preferences for female long-calls, as well as making their long-calls more female-like (Vicario et al., 2001). This suggests that RA, a motor nucleus whose primary function is the production of vocalizations, is also involved in the perception of long-calls. Young male zebra finches tend to respond like adult females to long-calls, and it was suggested that this might be due to the lack of fully mature connections between the nucleus HVC and RA (Vicario et al., 2001). There is evidence suggesting that nuclei in the song-control system play a role in the behavioural preferences for the female over the male long-call in zebra finches, and that matured connections between HVC and RA may be crucial.

The objective of this study was to determine if the song-control nucleus HVC plays a role in the perception of male and female long-calls in zebra finches, and if HVC modulates neural activity of the auditory forebrain; CMM and NCM specifically. HVC is involved in both the posterior descending pathway that is necessary for the acquisition and production of song, as well as the anterior forebrain pathway, which is necessary for acquisition only (Nottebohm, 2005). HVC also indirectly receives projections from auditory forebrain structures (e.g., CMM and NCM; Amador & Margoliash, 2011). Therefore, it seems likely that HVC modulates the neural responses of auditory forebrain

regions to female and male long-calls in zebra finches. I hypothesized that males without functional HVCs would respond similarly to females in response to both male and female long-calls, and would differ significantly from intact males. To test this hypothesis, I compared immediate-early gene (ZENK) responses in the auditory forebrain among six groups of birds: intact males who heard male long-calls, intact males who heard female-long-calls, intact females who heard male long-calls, intact females who heard female long-calls, HVC-lesioned males who heard male long-calls and HVC-lesioned males who heard female long-calls (see Table 5-1). I predicted that HVC-lesioned males and intact females would have similar levels of ZENK in response to male and female long-calls. Based on previous findings, I predicted that the HVC-lesioned males and females would show increased ZENK in NCM and CMM to the female long-call, whereas intact males would not (Gobes et al., 2009).

5.2 Methods

5.2.1 Subjects and housing

Starting in August 2016, a total of 36 zebra finches from the aviary colony at the Advanced Facility for Avian Research (AFAR) at the University of Western Ontario, London, Ontario, Canada, were used in this experiment. I pseudo-randomly assigned zebra finches to each of the 4 experimental conditions while accounting for sex of the individual, therefore a total of 8 experimental groups (see Table 5-1). Birds were kept in the aviary colony with ad libitum access to multi-vitamin seeds, grit, cuttlefish bones and water until they were moved to isolation for the experiment. Birds were identified as males or females by plumage. The room with the aviary colony was set at a 14 h light: 10 h dark cycle, which was mimicked in the modified audiometric testing booths. When the

experiment commenced, non-surgical zebra finches were placed into modified audiometric testing booths, whereas those in the surgery groups underwent either a sham or HVC-lesion surgery.

Table 5- 1 Sample sizes of experimental condition and playback conditions.

		Experimental Condition				
		Intact Female Zebra Finches	Intact Male Zebra Finches	Sham-lesioned Male Zebra Finches	HVC-lesioned Male Zebra Finches	Total Number of Birds
Playback Condition	Male Long-call	6	3	3	6	18
	Female Long-call	6	3	3	6	18
	Total Number of birds	12	6	6	12	36

5.2.2 Sham and HVC lesion surgery

Only male zebra finches were used for both the HVC lesion and sham surgeries. Female zebra finches were not lesioned; this is due to the fact that female zebra finches have a very small HVC, which may not be functionally connected to RA.

5.2.2.1 HVC lesion surgery

I injected birds intramuscularly with analgesic (0.01 mL of 0.625 mg/mL meloxicam). After the birds were anesthetized with 2.5% isoflurane at a flow rate of 2 L of oxygen per minute, I securely placed their heads in a stereotaxic mount, where a drill (Dremel) and 1-μL Hamilton syringe were mounted. I removed the feathers along the central part of the skull by using 70% ethanol, I disinfected the skin with a microbicide

(Betadine ®), and again applied 70% ethanol. I applied a small amount of topical local anesthetic (mix of lidocaine and prilocaine, EMLA® cream) to the skin. I made an incision of 0.75 cm in length along the midline and exposed the skull; I then positioned the drill bit at the tip of the central sinus that was used as the fronto-caudal marker for the stereotaxic coordinates. I moved the drill 2.5 mm lateral, and 0.1 mm rostral from the central sinus to the left hemisphere, and drilled a hole into the skull exposing the brain (see Figure 5-2). The coordinates were determined by using the zebra finch atlas and adjusted based on discussion with an expert (personal communication Marc Schmidt). I pierced through the meninges using a 26-gauge needle tip. I repeated the same procedure

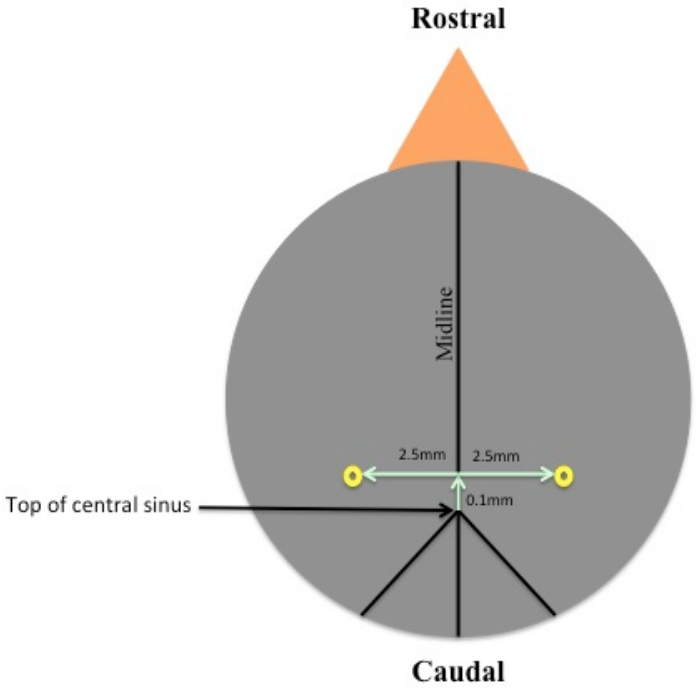


Figure 5- 2 Diagram of the zebra finch head during surgery. The midline and central sinus, which were used as markers for the stereotaxic measurements for the dremel placement, are depicted. The yellow circles show the locations where the skull was perforated with the drill and the Hamilton syringe was inserted. These measures were the same for the sham and HVC lesioned birds.

for the right hemisphere. I aligned the Hamilton syringe with the hole in the skull and lowered the syringe into the brain 2mm in depth, and then retracted to 1mm in depth. Over a period of 3-min I infused 0.2 μL of a glutamatergic neurotoxin (1% ibotenic acid in phosphate buffered saline; Sigma; St. Louis, Mo.). I retracted the Hamilton syringe and repeated the procedure in the right hemisphere. I then closed the skin using a tissue adhesive (3M Vetbond™), and returned the birds to their home cages inside individual isolation chambers, where they were allowed to recover for 3 days and received 0.01 mL of 0.625 mg/mL meloxicam each of the 3 days. One HVC-lesioned male died due to post-operative complications.

5.2.2.2 Sham surgery

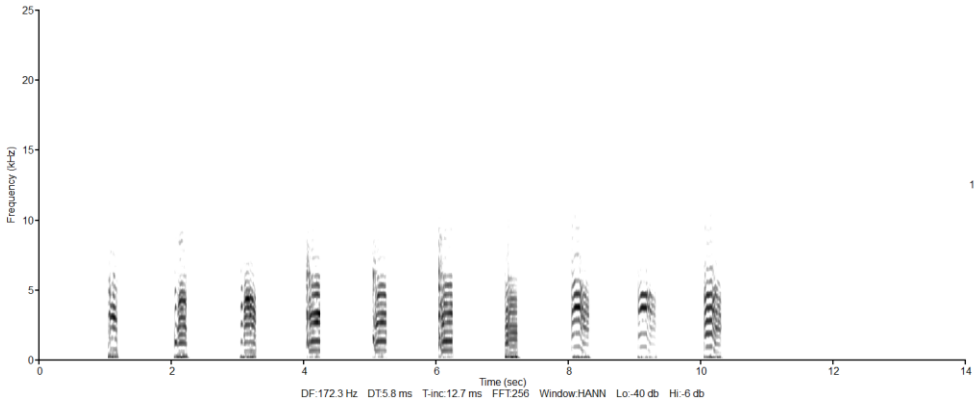
The birds in the sham lesion surgery group followed the same protocol as the one listed above until the point of where the holes were drilled into the skull. The holes were drilled into the skull and the meninges were pierced with a surgical needle tip, however the Hamilton syringe was not lowered into the brain. The bird remained under anesthesia for an additional 6-min (i.e., the time to infuse the ibotenic acid into both hemispheres), before closing the skin using a tissue adhesive (3M Vetbond™). I then returned the birds to their home cages inside the individual isolation chambers, where they were allowed to recover for 3 days and received 0.01 mL of 0.625 mg/mL meloxicam each of the 3 days.

5.2.3 Playback stimuli and playback procedure

Using a Marantz PMD 671 recorder attached to a Sennheiser microphone I recorded both female and male zebra finches in order to obtain audio samples of the female and male long-calls. The calls were confirmed as long-calls with the aid of Sharon M. H. Gobes (Wellesley College) and Marc Schmidt (University of Pennsylvania). Using

RavenPro 1.4 (Bioacoustics Research Program, 2011) I constructed two different types of audio stimuli; (1) female long-calls and (2) male long-calls (see Figure 5-3). The female and male long-calls had three different stimuli sets consisting of 10 vocalizations produced by at least five separate zebra finches, with no calls repeating between stimulus sets. Within each stimulus set the calls were repeated once per s for 10-s followed by 20-s of silence to form a 30-s sequence. This 30-s sequence was repeated 20 times to make a 10-min playback stimulus. Using the website, www.random.org, the order in which the vocalizations were presented was randomized for each 30-s stimulus set. Vocalizations were bandpass-filtered between 1000 and 22,000 Hz using RavenPro 1.4 (Bioacoustics Research Program, 2011) to remove background noise, and the amplitude was equalized across vocalizations.

A)



B)

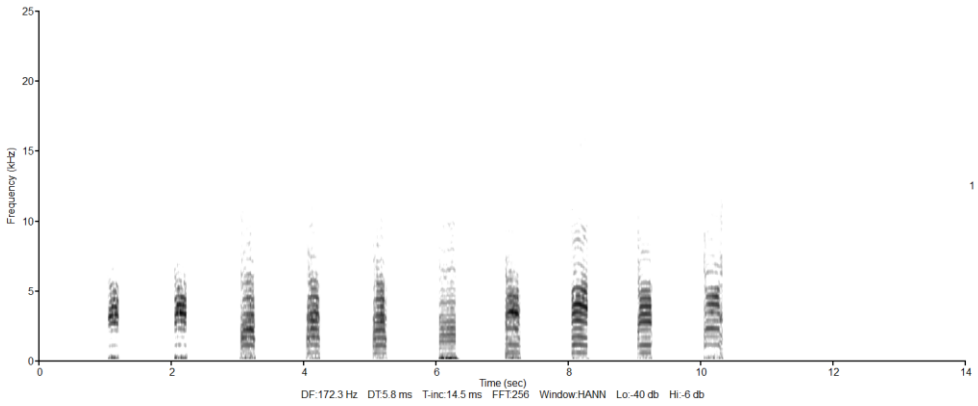


Figure 5- 3 Examples of the different vocalizations for the different playback groups. Each spectrogram represents the time in seconds on the x-axis and the frequency in kHz on the y-axis. Each vocalization is played followed by a 1 s period of silence before the next vocalization. This is repeated until the sample is approximately 10 s in length, and then followed by a 20 s period of silence and then repeated. A) Sample male long-call playback vocalizations, B) sample female long-call playback vocalizations.

5.2.4 Behavioural recordings and analyses

Birds in all conditions (i.e., intact females, intact males, sham-surgery males, and HVC lesioned males) were subjected to the same playback procedures. All birds were put into isolation for a minimum of 24 h prior to playbacks. I randomly assigned the zebra finches to one of the two playback conditions while ensuring a balanced sex ratio for the intact birds (see Table 5-1). I moved the birds into individual cages (25 cm × 30 cm × 37cm) inside a modified audiometric testing booth (width 91cm X height 172cm X depth 71cm, Industrial Acoustics Company, Inc., Bronx, NY). The photoperiod inside the isolation chamber matched the one from the aviary colony (14 h light: 10 h dark cycle). Prior to moving the individual bird into isolation, I outfitted each audiometric testing booth with one pair of speakers (Koss HDM/111BK) attached to a HipStreet (model HS-636-4GBBL) mp3 player located outside of the chamber, preventing the bird from being

disrupted when I began the playback treatments. I also installed a video camera to the ceiling of the chamber attached to a USB port outside the chamber to allow recording and verify that the bird was not vocalizing during the playback. Prior to the playback, the lights in the chamber were turned off for 1 h and remained off during the playback. The playback was started for 10 min, and the bird then remained in the silent and dark chamber for an additional 50 min (following Gobes et al., 2009).

For birds in all of the above groups, following the hour of isolation I anesthetized birds using isoflurane. Following deep anesthesia, birds were euthanized by transcardial perfusion with 0.1M phosphate buffered saline (PBS) followed by buffered 4% paraformaldehyde. I quickly removed the brain from the skull and placed it in 4% paraformaldehyde (~24 h) and then in 30% sucrose (~36 h) at 4 ˚C. Brains were frozen on crushed dry ice and then stored at -80 ˚C.

5.2.5 Nissl histology and quantification

Using a cryostat, I sectioned brains along the sagittal plane in 40-µm sections. I thaw-mounted every other section once the cerebellum was visible onto electrostatically treated microscope slides (VWR VistaVision™ Histobond ®). The slide was dried on a slide warmer for 5 min before being submerged in 4% paraformaldehyde for 5 min, and left to air-dry overnight before being processed the following day.

Once dry, the slides were stained using thionin, followed by serial dehydrations with increasing concentrations of ethanol, and cleared of lipids with an organic solvent (NeoClear, cat no. 65038-71; EMD Chemicals, Mississauga, Ontario, Canada). Finally the slides were covered with coverslips using a mounting medium (Permount, cat no.

SP15; Fisher Scientific) and allowed to dry in the fume hood ~ 24 h. I determined the location of HVC and the lesions by using a Leica DM 5500B microscope coupled to a Leica 420C camera. For each zebra-finch in the lesion condition, a minimum of 18 images (n = 11, M = 23.36, SD = 4.34) were captured using both the 1.25x and 5x objective lens, of all sections containing a lesion, as well as images of intact parts of HVC if available in the sections. The sections were selected such that the middle of the imaged section contained the largest cross-section of HVC with the lesion clearly visible. The lesions were therefore classified as either a 'hit' or a 'miss'. A hit was recorded if the lesion damaged at least part of the HVC in both hemispheres, whereas a miss was recorded if no part of HVC was damaged in either hemisphere. The lesions were then classified into 2 categories; hit/hit (n = 12), and miss/miss (n = 6). The location of the lesions for all successful lesions was then traced on images retrieved from the ZEBrA database.

A lesion was considered successful if it had affected HVC in both the left and right hemisphere. Neurochemical lesion studies have shown that the location of the lesion within HVC doesn't affect the effectiveness at producing behavioural effects; rather it is the integrity of HVC itself that matters (Del Negro, Gahr, Leboucher, & Kreutzer, 1998).

5.2.6 ZENK immunohistochemistry

I ran immunohistochemistry in multiple runs counterbalanced across the different playback and surgical groups. I used an established immunohistochemistry protocol where multiple sections were contained in wells in 24-well tissue-culture trays, and the solutions were pipetted in and out of each individual well (Farrell, Neuert, Cui, &

MacDougall-Shackleton, 2015; Hernandez & MacDougall-Shackleton, 2004; Maney, MacDougall-Shackleton, MacDougall-Shackleton, Ball, & Hahn, 2003; McKenzie, Hernandez, & MacDougall-Shackleton, 2006; Schmidt, McCallum, MacDougall-Shackleton, & MacDougall-Shackleton, 2013). Using a cryostat, I sectioned brains along the sagittal plane in 40 µm sections and temporarily stored in 0.1M PBS. Every second section (i.e., 80 µm) was used to examine ZENK immunoreactivity (ZENK-ir) and one series was saved as back-up for birds in the intact female and male groups. First, free-floating sections were thoroughly rinsed twice with 0.1M PBS, and then incubated with 0.5% H_2O_2 in PBS for 15-min to eliminate endogenous peroxidase activity. Sections were washed three times with 0.1 M PBS, and then incubated in 10% Normal Goat Serum (cat no. S-1000; Vector Laboratories, Burlingame, CA USA) in 0.1 M PBS containing 0.3% Triton X-100 (0.3% PBS/T) for 1 h. Sections were then incubated with primary antibody made in rabbit against Egr-1 (polyclonal, 1:4000, cat no. SC-189; Santa Cruz Biotechnology, Santa Cruz, CA USA) in 0.3% PBS/T for ~24 h at 4 ˚C. After rinsing three times with 0.1% PBS/T, sections were incubated with biotinylated goat anti-rabbit IgG secondary antibody (1:250 dilution) for 1 h at room temperature, followed by three rinses with 0.1% PBS/T. Sections were then incubated with avidin-biotin horseradish-peroxidase complex (VectaStain Elite ABC Kit, cat no. PK 6100; Vector Laboratories) at dilution 1:200 for 1 h, followed by two rinses with 0.1% PBS/T. The tissue sections' immunoreactivity was then visualized with 3, 3'-diaminobenzidine tetrahydrochloride (SigmaFAST DAB, cat no. D4418; Sigma). After thoroughly rinsing the sections with PBS, I mounted the sections onto electrostatically treated microscope slides (VWR VistaVision™ Histobond ®) and let them dry overnight. Once dry, I put the slides

through serial dehydrations with increasing concentrations of ethanol, and cleared of lipids with an organic solvent (NeoClear, cat no. 65038-71; EMD Chemicals, Mississauga, Ontario, Canada). Finally slides were covered with coverslips using a mounting medium (Permount, cat no. SP15; Fisher Scientific) and allowed to dry in a fume hood ~12 h.

5.2.7 ZENK quantification

For each field of interest, z-stack images of automatic step size through the focal planes were collected through the 20× objective lens and were then compiled using a montage mode in Leica Application Suite software. This allowed for all the ZENK-ir cells to be in focus within the same image. I used Leica Application Suite to compile each picture as a z-stack from a series of images taken at a regular intervals (0.63 µm) throughout the focal depth of the section using a Leica 420D camera. Compiling these photomicrographs created an image in which all cells were in focus (Hall & MacDougall-Shackleton, 2012). For each image, the area (mm^2) was determined by using a calibration image also taken with the 20× objective lens. For each image, I used ImageJ64 (NIH) software to count the number of ZENK-ir cells in the whole image. First, I converted the images to 8-bit gray scale, then the number of particles with an optical density above a threshold value were counted using the threshold tool. This threshold was set manually in every image due to the variability in the background staining, in a way that the group of pixels emphasized by the software were equivalent with what a blind observer considered labeled nuclei. To set exclusion limits for cell size (2.0 – 56 μm^2) I randomly selected 6 birds and from the 18 photomicrographs per bird (6 x each area) and chose a subset of 20 cells. From these 360 measurements per bird, 2,160 measurements in total, I determined

the minimum and maximum sizes of the cells and established a minimum and maximum. Exclusion limits for sphericity were set at 0.45.

ZENK immunoreactivity (ZENK-ir) was quantified for three auditory regions: CMM, dorsal NCM (NCMd) and ventral NCM (NCMv; Figure 5-4) by using a Leica DM

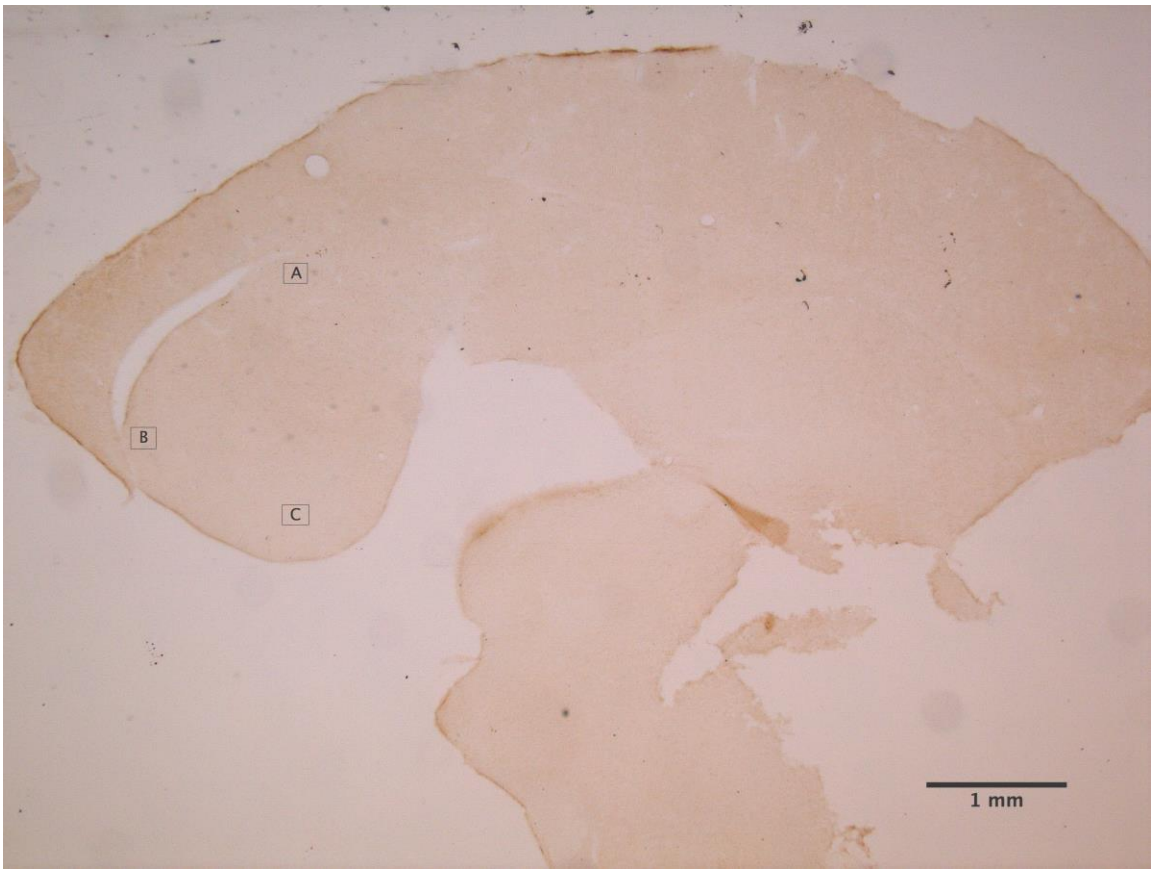


Figure 5- 4 Sagittal slice of zebra finch auditory forebrain. Sampling region used to quantify ZENK-ir in CMM (A), NCMd (B) and NCMv (C). Left is caudal and right is rostral. The boxes are not representative of the actual scale of the sampling area, but are to demonstrate the location where the images were taken.

5500B microscope coupled to a Leica 420C camera. For each zebra finch 10 to 12 images were captured, six sections of one hemisphere of each zebra finch for CMM, NCMd, and

NCMv. I began quantification with the first section, moving medial to lateral, where NCM was attached to the rest of the brain. Therefore, six photomicrographs per area, per bird were taken. For NCMd the photomicrographs were taken from the most dorso-caudal part of NCM. NCMv photomicrographs were obtained from the center of the ventro-rostral area. CMM photomicrographs were acquired from the most caudal part of the structure. In all three forebrain auditory regions we captured images from the areas with the highest density of immuno-positive ZENK cells within the area (see Figure 5-4)(following Avey, Phillmore, & MacDougall-Shackleton, 2005; Gentner, Hulse, Duffy, & Ball, 2001; Hernandez & Macdougall-shackleton, 2003; Schmidt, McCallum, MacDougall-Shackleton, & MacDougall-Shackleton, 2013). For each image, the area (mm^2) was determined by using a calibration image also taken with the 20× objective lens. The measurements for area (mm^2) and cell counts were entered in a spreadsheet and the number of cells/mm^2 was determined.

5.2.8 Data and statistical analyses

Statistical analyses were carried out using IBM SPSS Statistics 24.0. The mean number of cells/mm^2 for each individual was compared between the right and left hemispheres using a paired t-test. No significant differences were found between hemispheres, therefore all analyses were conducted on the mean cell count pooled across hemispheres.

I first ran a 3-way repeated measures ANOVAs and 2-way repeated measures ANOVAs to examine the effects of the factors brain region (CMM, NCMd, NCMv), playback stimulus (female or male long-call), and experimental condition were

significant (intact female, intact males, and HVC lesioned males). HVC lesion locations was not determined prior to the playback of the different vocalizations, and because three birds received bilateral HVC lesions that completely spared HVC these birds were put into the "intact male" control group. One bird also died as a result of complications (excessive bleeding) during surgery. The results were qualitatively the same whether these individuals were removed entirely from the analyses, or if they were included as "intact males". For post-hoc analyses six t-tests were run to determine if there were differences between the ZENK-ir in CMM, NCMd and NCMv for the male and female long-calls for the intact compared to HVC-lesioned males. Another 6 t-tests were conducted comparing the differences for male and female long-calls for HVC lesioned males as well as intact males. Results were considered significant at $\alpha \leq 0.05$ level. Data are presented as mean ± SEM.

Table 5- 2 Sample sizes of experimental condition and playback conditions after HVC lesions were verified.

		Experimental Condition			
		Female Zebra Finches	Intact Male Zebra Finches	HVC Lesioned Zebra Finches	Total Number of Birds
Playback Condition	Male Long-Call	6	8	3	17
	Female Long-Call	6	7	5	18
	Bird Total number	12	15	8	35

5.3 Results

5.3.1 3-way ANOVA

No significant differences were found between the sham-lesioned males and the intact males, $p > 0.05$, therefore their data were combined into one group for intact males.

Table 5- 3 Results of the 3-way ANOVA.

	Variable	F	df	p
Within-subjects effects	Brain Region	60.712*	2, 58	<0.001
	Brain Region X Experimental Condition	0.751	4, 58	0.56
	Brain Region X Playback Condition	0.555	2, 58	0.58
	Brain Region X Experimental Condition X Playback Condition	1.569	4, 58	0.20
Between-subjects effects	Experimental Condition	0.281	2, 29	0.76
	Playback Condition	2.049	1, 29	0.16
	Experimental Condition X Playback Condition	3.973*	2, 29	0.03

CMM showed the greatest number of ZENK-ir cells, followed by NCMd, and lastly NCMv (see Figure 5-5). No significant interactions were found between brain region and any other factors. In addition to the significant main effect of brain region, there was a significant interaction between experimental group and playback condition (see Table 5-3). Although there was no significant overall main effect of treatment group or playback condition the significant interaction indicates that birds in different groups had different patterns of response to male versus female long-call playback. To explore this interaction further, I conducted post-hoc ANOVA on each group separately.

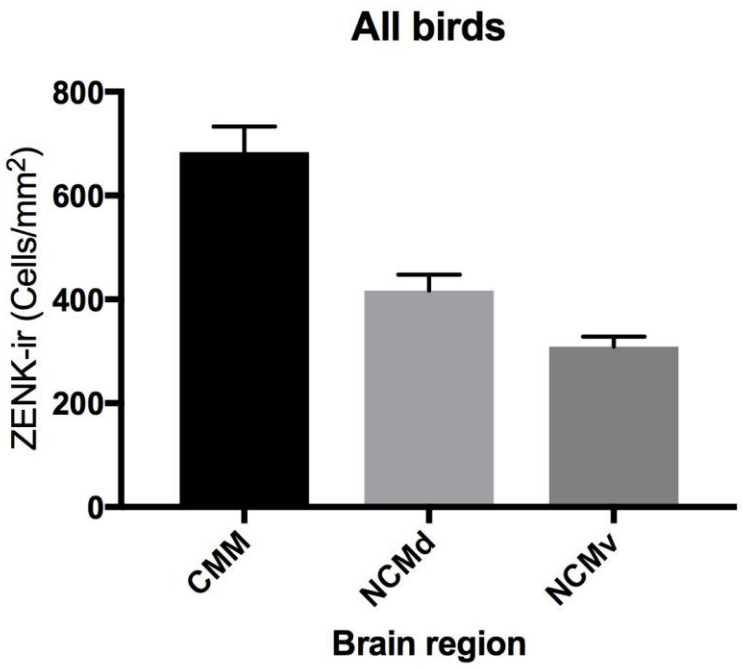


Figure 5- 5 Differences in ZENK-ir across the three auditory telencephalon regions, CMM, NCMd, NCMv. The zebra finches displayed the most ZENK-ir cells in CMM, followed by ZENK-ir cells in NCMd, and showing the least ZENK-ir cells in NCMv.

5.3.2 Intact Males

For intact males there was only a main effect of brain region, there were no differences between the playback conditions in any of the auditory forebrain structures, CMM, NCM, NCMv (see Table 5-4, Figure 5-6). There were no significant differences in ZENK-ir between female and male long-calls for CMM, and NCMv ($p > 0.05$) for intact lesioned males.

Table 5- 4 Results for ZENK-ir in the auditory forebrain for intact male zebra finches.

	Variable	F	df	p
Within Subjects Effects	Brain Region	27.626*	2, 26	<0.001
	Brain Region X Playback Condition	0.742	2, 26	0.05
Between Subjects Effects	Playback Condition	1.690	1, 13	0.22

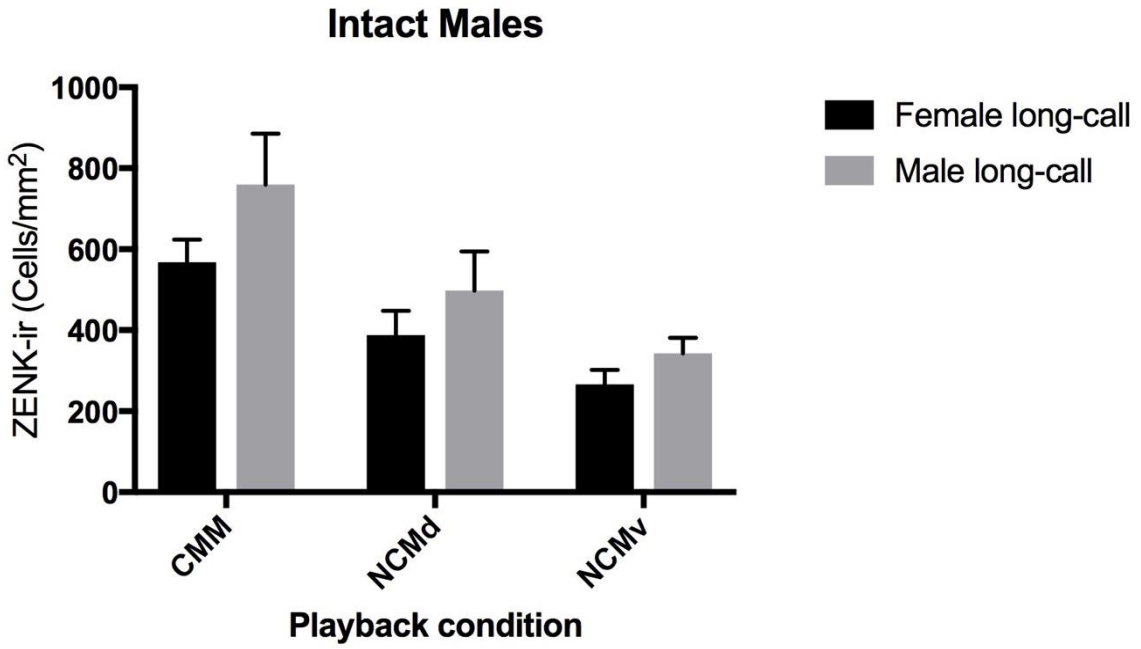


Figure 5- 6 The differences in ZENK-ir cells in response to playbacks of female and male long-calls in intact males across the auditory telencephalon. There are no significant differences between female and male long-call playbacks in intact males.

5.3.3 Intact Females

For intact females there was only a main effect of brain region, there were no differences between the playback conditions in any of the auditory forebrain structures, CMM, NCM, NCMv (see Table 5-5, Figure 5-7).

Table 5- 5 Results for ZENK-ir in the auditory forebrain for intact female zebra finches.

	Variable	F	df	p
Within Subjects Effects	Brain Region	23.770*	2, 20	<0.001
	Brain Region X Playback Condition	0.955	2, 20	0.40
Between Subjects Effects	Playback Condition	2.805	1, 10	0.22

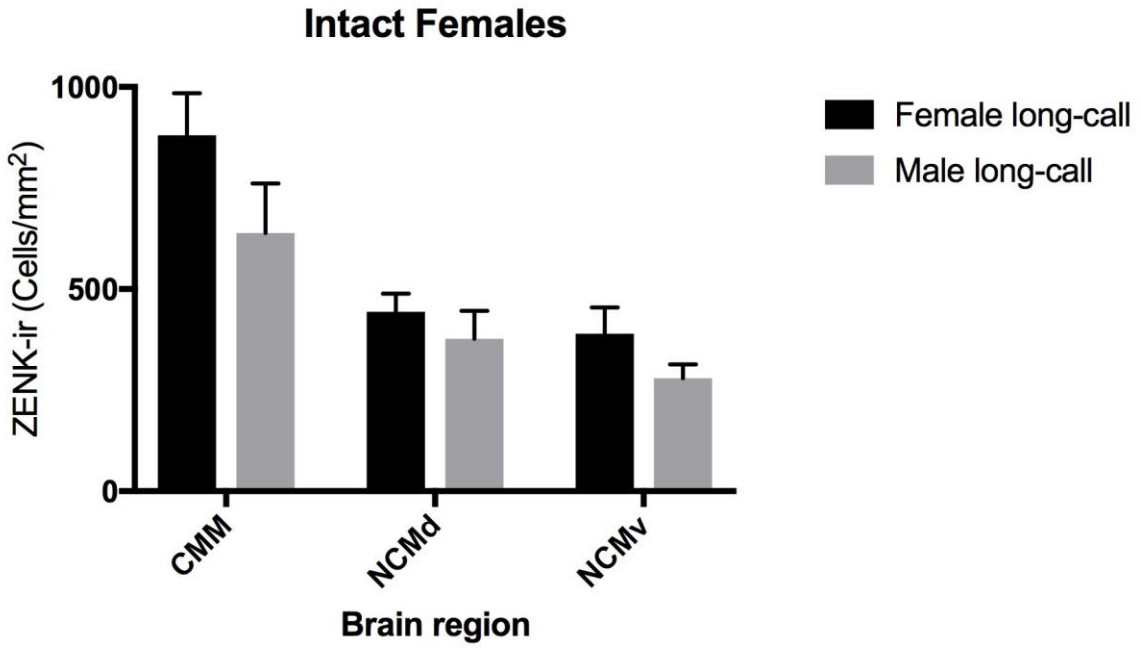


Figure 5- 7 The differences in ZENK-ir between female and male long-call playbacks in intact females across the auditory telencephalon. There are no significant differences between female and male long-call playbacks in intact females.

5.3.4 HVC lesioned males

For HVC lesioned males, there was a significant effect of playback condition; there was more ZENK-ir for the male long-call than the female long-call across all auditory forebrain structures, and there was also a main effect of brain region (see Table 5-6, Figure 5-8). There were no differences in ZENK-ir for CMM, NCMd and NCMv for

intact compared to HVC lesioned males for either playback condition (all $p > 0.05$). However there were significant differences in ZENK-ir between female and male long-calls for CMM ($t(14) = 3.21$, $p = 0.006$), NCMd ($t(14) = 3.07$, $p = 0.008$) and NCMv ($t(14) = 3.01$, $p = 0.009$) for HVC lesioned males.

Table 5- 6 Results for ZENK-ir in the auditory forebrain for HVC lesioned male zebra finches.

	Variable	F	df	p
Within Subjects Effects	Brain Region	16.850*	2, 12	<0.001
	Brain Region X Playback Condition	2.194	2, 12	0.15
Between Subjects Effects	Playback Condition	7.018*	1, 6	0.04

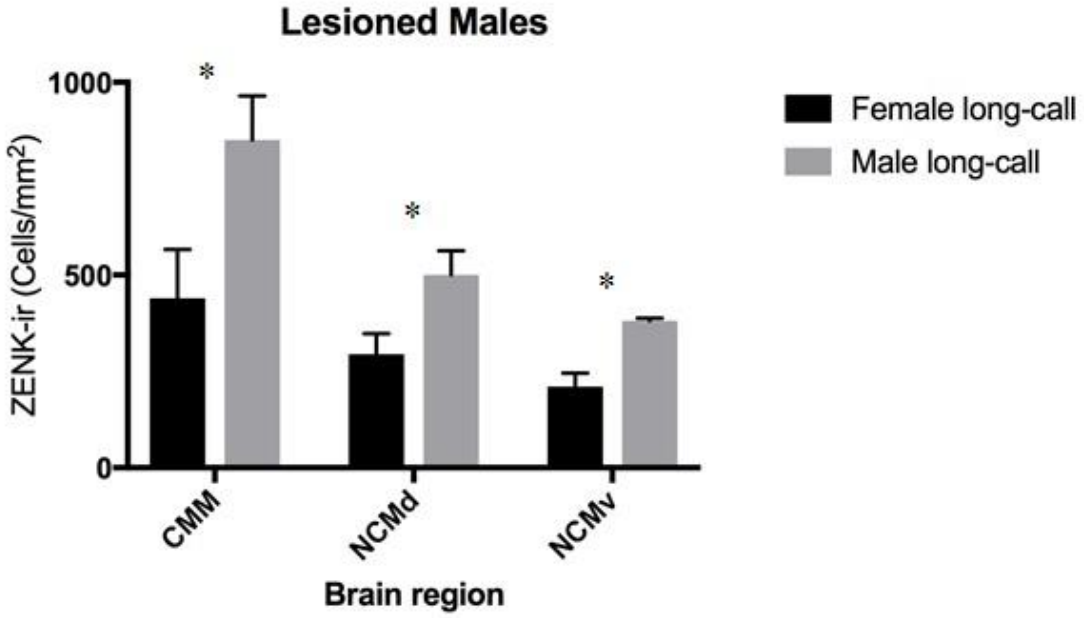


Figure 5- 8 The differences in ZENK-ir between female and male long-call playbacks in HVC lesioned males across the auditory telencephalon. There is more ZENK-ir for the male long-call in CMM, NCMd and NCMv.

5.4 Discussion

This study was conducted in order to determine if HVC plays a role in the perception of male and female long-calls in zebra finches, and specifically if this role is reflected in the neural activity of the auditory forebrain, CMM and NCM. The data does support the conclusion that HVC is involved in the perception of the female and male-long-calls in male zebra finches. Neither male nor female zebra finches showed a significant difference in their neural responses to female or male long-calls, whereas HVC lesioned males showed more neural activation for male long-calls compared to female long-calls, indicating that HVC may be involved in some auditory processing which equates male and female calls, which does not occur when HVC is no longer active. Overall there was also the most ZENK-ir cells in CMM, followed by NCMd, and NCMv, which is consistent with a trend that is observed when moving down the auditory pathway and is in accordance with previous songbird ZENK-ir studies (Avey, Kanyo, Irwin, & Sturdy, 2008; Phillmore, Bloomfield, & Weisman, 2003).

5.4.1 Intact Males and Females

Intact males did not show a difference in ZENK-ir cells in the auditory forebrain between male and female long-calls. The females also did not show a different neural response to female and male long-calls. These results are similar to ones obtained by Gobes and colleagues (2009); they did not find any differences for female and male zebra finches in ZENK-ir in CMM and NCM for male or female long-call playback. The only difference they obtained was the females showed increased ZENK-ir in the auditory regions compared to females who only heard silence. These results may not be surprising, as previous electrophysiological studies have examined the responsiveness of neurons in

NCM to auditory stimuli of calls and songs and found no differences in response rates between males and females (Chew et al., 1996). This could mean that processing of these auditory stimuli may be occurring in higher-order structure like HVC, which has reciprocal projections from a subdivision of CMM (nucleus avalanche) and HVC (Akutagawa & Konishi, 2010; Lewandowski, Vyssotski, Hahnloser, & Schmidt, 2013; Nottebohm, Kelley, & Paton, 1982). Because long-calls are used to maintain contact when birds are visually separated from one another, it is possible that they process unfamiliar male and female long-calls similarly. We know that zebra finches recognize long-calls of their mates, or of their social group (Forstmeier, Burger, Temnow, & Deregnaucourt, 2009; Giret, Menardy, & Del Negro, 2015; Vignal & Mathevon, 2011; Vignal et al., 2004). The stimuli used in this study were not calls that would have been from individuals in the same colony as the birds tested, therefore it may be likely that long-calls were processed similarly. It could have been processed as a call from a member of the same species, without further processing that may occur when a call is more familiar to the zebra finch.

5.4.2 HVC lesioned males

Lesioned males showed a significant difference in their ZENK-ir cells in the auditory forebrain in response to female and male long-calls. HVC lesioned males showed more ZENK-ir cells for male long-calls than female long-calls in the auditory forebrain. HVC and RA have both been shown to be crucial in the production of male-typical long-calls, where without functioning HVC and RA, the male long-call loses its male typical attributes such as the frequency modulation, and becomes longer, therefore much more female like (Simpson & Vicario, 1990). It was therefore very likely that HVC

may also be involved in the processing of the long-call due to the reciprocal connections between a subdivision of CMM known as nucleus avalanche and HVC (Akutagawa & Konishi, 2010; Lewandowski et al., 2013; Nottebohm et al., 1982). Because this connection is reciprocal it is possible that HVC processes the auditory stimuli and deems the long-calls from males and females as having equivalent valence. It is possible that HVC may play a role in the transformation of a signal, encoding the salience of the stimulus parameters into a control signal that modulates the neural auditory processing of the long-call. In intact birds, long-calls from unfamiliar zebra finches may be processed the same way by males and females, whereas in HVC lesioned males may process them in an altered way, showing more neural activation for male long-calls than female ones. Especially since CMM and NCM are secondary auditory regions which are involved in some of the processing of complex vocal signals (Amador & Margoliash, 2011; Vates, Broome, Mello, & Nottebohm, 1996)

It would be interesting to investigate the perception of female and male long-calls in juvenile zebra finches, since the connections between RA and HVC have yet to mature. And we know that the lesioning of RA in male zebra finches affects their behavioural preferences for female long-calls, as well as making their long-calls more female-like in their structure (Vicario et al., 2001). Because the connections between HVC and RA require time to fully mature, it might be possible to also see a difference in the neural perception of unfamiliar female and male long-calls in juvenile zebra finches. It would be interesting to examine whether the reciprocal connection between nucleus avalanche in CMM to HVC is mature in young zebra finches as well.

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Chapter 6

6 General Discussion

6.1 The song-control system and call production

In this thesis, my main objective was to investigate the neural mechanisms that underlie the production and perception of bird calls, specifically examining candidate structures within the song-control system. My first objective was to understand the role of the song-control system in the production of bird calls. I investigated this in two experiments. In Chapter 2, I examined neural activity in the song-control system of the black-capped chickadees during the production of their *fee-bee* song, *chick-a-dee*, *gargle* and *tseet* calls. I found that the *gargle* call was associated with the most ZENK gene expression in HVC and the robust nucleus of the arcopallium (RA). The activation also scaled with the complexity of the vocalization (defined as a vocalization with more notes, more rapid frequency modulations and larger frequency ranges), with the *gargle* call having the most, and the *tseet* call having the least, immediate-early gene induction. Therefore more neurons were firing in HVC and RA during the production of more complex vocalizations, compared to simpler ones that the black-capped chickadee produces.

The results of Chapter 2 indicated that HVC is a crucial structure for call production, however the proportion of the immediate-early gene response driven by motor activity, as opposed to auditory feedback, was not clear. Therefore, in Chapter 3, I inactivated HVC in both hemispheres of the brain with an excitotoxic lesion, and examined the effects this had on the *gargle* and *chick-a-dee* calls of black-capped chickadees. The *gargle* calls were negatively impacted by the bilateral HVC lesions, they

were much more inconsistent, often missing parts of notes, entire notes and whole portions of the calls. The bioacoustics measures (e.g., duration, frequency, etc.) also supported these results. The HVC lesions also affected the *chick-a-dee* calls, particularly the A, B and D notes. Therefore I conclude that HVC is not only crucial for call production but it plays a role in the production of particular acoustic structures, note types and other characteristics of the *gargle* and to a lesser extent the *chick-a-dee* calls. Thus HVC is not only a song-control nucleus, but is required for the production of a variety of complex, and potentially learned, vocalizations.

Although the involvement of HVC in call production in black-capped chickadees was previously unknown, similar evidence had been demonstrated in zebra and Bengalese finches (Halle, Gahr, & Kreutzer, 2003; Simpson & Vicario, 1990, 1991; Ter Maat, Trost, Sagunsky, Seltmann, & Gahr, 2014; Urbano, Aston, & Cooper, 2016). Zebra finches with lesions to HVC show similar deficits in their long-call that chickadees show in their *gargle* calls. The male long-call is partially learned, and when HVC or RA is lesioned, the long-call resembles the innate long-call of a female zebra finch (Simpson & Vicario, 1990). In zebra finches, HVC lesions change the fundamental frequency, the fast frequency modulations and the temporal structure of the long-call, which are the more complex portions of the male long-call (Price, 1979; Simpson & Vicario, 1990; Zann, 1984, 1985). This is very similar to bioacoustic effects on the *gargle* calls after bilateral HVC lesions of black-capped chickadees in my study. Single-hemisphere HVC lesions also affect the bioacoustic frequency measures (e.g., decrease in top frequency or increase in the lowest frequency) of the long-call in zebra finches (Halle et al., 2003). Similarly, the chickadees showed changes in these frequency measures for the *gargle*

call. Therefore HVC is not only crucial for song learning and production but also for calls as well, in particular for the learned, acoustically complex portions of calls.

One limitation of the studies above is that they do not allow us to understand what is going on within the brain in real time. In-vivo electrophysiology would allow us to understand how the neuronal firing rates, and the different neuron types, are involved in call production. In zebra finches, neurons within RA fire during the production of *tet stack* calls, which is a very simple vocalization that zebra finches produce (Ter Maat et al., 2014). Although this call is simple, it does require the involvement of RA in order to produce it correctly. Although I found much less ZENK-ir in RA than HVC for the production of all the vocalizations, this structure may also be integral for the production of all calls the black-capped chickadee produces.

6.2 Neural basis of perception of bird calls

My second objective for this thesis was to understand how bird calls were perceived in the brain. In Chapter 4 I used ZENK gene expression to determine if there is a difference in the neural processing of the *fee-bee* song, *gargle* and *chick-a-dee* calls in the auditory forebrain of black-capped chickadees. I found that the *gargle* call elicited the most ZENK response in CMM, ventral NCM and dorsal NCM, which are all components of the auditory forebrain. These differences in immediate-early gene response could be mediated by the complexity of the vocalization and not the function of the vocalization (Hernandez et al., 2008). The most complex vocalization, the *gargle* call, elicited the most immediate-early gene response, followed by less expression for the *chick-a-dee* call, and even less for the *fee-bee* song.

There are two plausible functions of CMM, NCMd, and NCMv in the neural response to different chickadee vocalizations; 1) that the function of the call is driving the neural response in the auditory forebrain or 2) that the complexity of the call is driving the neural response in the auditory forebrain. Previous contradictory results of ZENK gene expression in the auditory forebrain when chickadees are presented with *chick-a-dee* calls and *fee-bee* songs do not allow us to differentiate these possibilities (Avey, Kanyo, Irwin, & Sturdy, 2008; Phillmore, Bloomfield, & Weisman, 2003). Because the *fee-bee* song is used for territory defense and to attract mates, it is considered to have more function in reproduction (a defining feature of birdsong) than the *chick-a-dee* call. I found that the *gargle*, a more acoustically complex vocalization than the *fee-bee*, elicited much more ZENK response in the auditory forebrain. This was not entirely surprising because female starlings show much more ZENK gene expression to longer and more complex songs compared to simpler one (Gentner, Hulse, Duffy, & Ball, 2001). Therefore it seemed likely that this difference in neuronal response was due to call complexity, as the neural response decreases as the vocalization decreased in complexity. Similarly, chickadees who hear song-sparrow songs show increased ZENK gene expression in the auditory forebrain (Phillmore, Veysey, & Roach, 2011). Song-sparrow song is complex, with notes that modulate in frequency rapidly, which is similar to *gargle* note composition. This would again suggest that the auditory forebrain of the chickadee has more neurons firing when presented with more complex vocalizations, because the song-sparrow song would have little meaningful significance to the black-capped chickadee.

Overall my results suggest that the auditory forebrain and the song-control system would be heavily involved in the processing and production of more complex

vocalizations, like the *gargle* call, in black-capped chickadees. It would be interesting to test the hypothesis that the auditory forebrain has more neurons firing during more complex acoustic stimuli by presenting the chickadees with normal *gargle* calls as well as *gargle* calls that were produced post-lesion in Chapter 3. If the auditory forebrain is tuned to more complex acoustic stimuli we should observe more neural response to intact *gargles* than to HVC lesion *gargles*, as these are simpler. Therefore they may be deriving more information from the more complex information than from simpler ones.

6.3 The song-control system and the neural basis of perception of bird calls

My last objective in this thesis was to understand how the song-control system is involved in the perception of calls. Prior work suggests that HVC is involved in perceptual processing of birdsong in canaries (Brenowitz, 1991) but not in female zebra finches (MacDougall-Shackleton, Hulse, & Ball, 1998). I wanted to understand the role that HVC plays in the perception of learned calls, specifically the long-calls in male zebra finches. Therefore in Chapter 5, I used excitotoxic lesions to inactivate HVC in both hemispheres and examined how this affected female and male long-call neural processing in the auditory forebrain. I found that intact male and female zebra finches did not show differences in ZENK response in auditory forebrain, however the HVC-lesioned zebra finches had more ZENK response to male long-calls compared to female long-calls. HVC has reciprocal connections with a subsection of CMM called the nucleus avalanche, which explains why we see auditory processing effects when HVC is lesioned (Akutagawa & Konishi, 2010; Lewandowski, Vyssotski, Hahnloser, & Schmidt, 2013; Nottebohm, Kelley, & Paton, 1982).

The results obtained could be interpreted to indicate that HVC is involved in higher-order processing of vocalizations. Because the female and male long-calls are used in the same context, for the same purpose, when these calls reach CMM they are further processed by HVC, which processes both calls as having equivalent valences. Without an active HVC, the processing must rely on the bioacoustic properties of the long-call, which in males is more complex. Therefore the more complex vocalization shows more ZENK gene expression in CMM and NCM, which are secondary auditory regions involved in some of the processing of complex vocal signals (Amador & Margoliash, 2011; Vates, Broome, Mello, & Nottebohm, 1996).

6.4 General Conclusions

Overall I set out to better understand the role of the song-control system in call production, as well as in call perception. I also set out to better understand how calls are perceived in the auditory forebrain. I found that HVC was not only crucial for call production, but also for how calls are perceived by the brain. Black-capped chickadees were primarily used because of the variety of complex calls they produce in addition to a very simple *fee-bee* song.

It is possible that the results obtained may be black-capped chickadee specific, although this is unlikely. Siberian tits (*Poecile cinctus*) have been shown to use *gargle* and *chick-a-dee* calls instead of song in a variety of situations where black-capped chickadees would produce the *fee-bee* song (Hailman, Haftorn, & Hailman, 1994). Similarly, black-capped chickadees have a greater neural response to more complex vocalizations, like a song-sparrow song, than to simpler vocalizations (Phillmore et al., 2011). This is similar to what is observed in female starlings, another songbird species,

who exhibit increased immediate-early gene expression in the auditory forebrain when presented with longer, more complex, male songs (Gentner et al., 2001).

Differences in the size of the song-control nuclei have been well documented, where often the size of the song-control nuclei tend to be larger in species with more complex songs, and that HVC is larger in individuals with a larger repertoire (Devoogd, Krebs, Healy, & Purvis, 1993). Many temperate-zone songbird species tend to sing primarily during the spring, when mating and breeding occur. Therefore many species show a seasonal variation in the volume of some or all of the song nuclei (Arai, Taniguchi, & Saito, 1989; Brenowitz, Nalls, Wingfield, & Kroodsma, 1991; Caro, Lambrechts, & Balthazart, 2005; Dloniak & Deviche, 2001; Kirn, Clower, Kroodsma, & Devoogd, 1989; Meitzen & Thompson, 2008; Nottebohm, 1981; Smith, Brenowitz, Wingfield, & Baptista, 1995; Smith, 1996). Although a related species, the Corsican blue tit (*Cyanistes caeruleus ogliastrae*), shows seasonal growth in HVC and RA, this is not the case in black-capped chickadees, although photostimulation does induce changes in the song-control system when the chickadee is in breeding condition (Smulders et al., 2006, but see MacDougall-Shackleton et al. 2003, Phillmore et al. 2006). This could be due to black-capped chickadees producing more complex calls throughout the year, like the *gargle* and *chick-a-dee* calls, which in turn require the year-round involvement of HVC to produce and perceive them. It is possible that because black-capped chickadees possess a repertoire of *gargle* calls, and not a repertoire of songs, that this may require the constant recruitment of neurons within HVC, which would explain why we do not see these seasonal changes in the song-control nuclei.

Based on the results obtained throughout this thesis, it is possible that the distinction between songs and calls is irrelevant when considering the activity of the song-control system, and the determining factors for neural activity in HVC is the complexity of the vocalization, and if it requires learning in order to produce it. The distinction between songs, which are vocalizations used to attract potential mates and defend territories, and calls, which are used for everything else, may be irrelevant in terms of motor control of the syrinx. The neural activity of HVC during the production of vocalizations may be based on complexity and learning. With regard to the song-control system, it seems only reasonable that the nuclei within the song-control system are involved in the production and perception of calls based on the results obtained. Therefore the song-control system is not aptly named, it should be referred to as the vocal-control system, as it is involved in call production and perception as well as song learning, production and perception.

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Appendices

Appendix A: Effects of HVC lesions on *gargle* calls. T-tests are provided for pre- and post-lesion comparisons of bioacoustic measurements. PC = percent change in the parameter, provided as an estimate of effect size of the lesion.

Bird lB.Bl HVC lesion (hit/hit lesion)

Table A- 1 Bird lB.Bl: Call 11

	Duration	Start Frequency	End Frequency	Top Frequency	Peak Frequency	F_{max}	f_0	NPF
Total call	**t(9)=6.713** PC=42.92**							
Note 1	t(9)=-.629 PC=-14.23	t(9)=-1.983 PC=-4.49	t(9)=-.911 PC=-18.95	t(9)=-.850 PC=-3.06	t(9)=-.796 PC=-3.04	t(9)=-1.022 PC=-3.01	N/A	N/A
Note 2	t(9)=-.788 PC=-6.50	t(9)=-.481 PC=-3.32	**t(9)=-2.830* PC=-16.24**	N/A	t(9)=.537 PC=1.06	t(9)=-.217 PC=-0.50	N/A	N/A
Note 3	t(9)=.961 PC=9.36	**t(9)=-25.699** PC=-49.79**	**t(9)=-2.722* PC=-83.25**	t(9)=.788 PC=16.63	**t(9)=3.930* PC=5.70**	**t(9)=3.900* PC=5.94**	N/A	N/A
Note 4	t(9)=2.080 PC=25.75	N/A	N/A	N/A	N/A	**t(9)=-2.378* PC=-14.65**	t(9)=-1.758 PC=-41.79	t(9)=.552 PC=8.71
Note 5	**No notes post-lesion**	**No notes post-lesion**	**No notes post-lesion**	**No notes post-lesion**	**No notes post-lesion**	**No notes post-lesion**	N/A	N/A

Table A- 2 Bird lB.Bl: Call 12

	Duration	Start Frequency	End Frequency	Top Frequency	Peak Frequency	F_{max}	f_0	NPF
Total call	t(9)=1.384 PC=20.94							
Note 1	t(9)=-1.237 PC=-46.42	**t(8)=3.290*** **PC=-9.74**	t(8)=-.687 PC=-9.55	t(8)=-.065 PC=-0.21	t(8)=-1.520 PC=-4.78	t(8)=-1.430 PC=-4.68	N/A	N/A
Note 2	**t(9)=2.989*** **PC=-17.80**	t(9)=-.777 PC=-6.13	t(9)=-.981 PC=-5.13	N/A	t(9)=.534 PC=0.96	t(9)=.885 PC=1.49	N/A	N/A
Note 3	t(9)=-1.080 PC=-27.63	t(9)=.958 PC=1.30	t(9)=-.267 PC=-7.42	t(9)=.102 PC=1.00	t(9)=1.365 PC=25.24	t(9)=1.371 PC=25.52	N/A	N/A
Note 4	t(9)=.492 PC=22.81	N/A	N/A	N/A	N/A	t(8)=.315 PC=5.55	t(8)=.184 PC=2.59	t(8)=1.701 PC=22.65

Table A- 3 Bird lB.Bl: Call 13

	Duration	Start Frequency	End Frequency	Top Frequency	Peak Frequency	F_{max}	f_0	NPF
Total call	t(2)=2.259 PC=11.62							
Note 1	t(2)=1.090 PC=36.62	t(2)=-1.606 PC=-6.11	t(2)=-1.223 PC=-35.12	t(2)=.000 PC=0.00	t(2)=.095 PC=0.78	t(2)=-.307 PC=-2.58	N/A	N/A
Note 2	t(2)=-.368 PC=-5.81	t(2)=.296 PC=3.48	t(2)=-3.645 PC=-23.87	N/A	t(2)=.896 PC=2.87	t(2)=1.070 PC=3.35	N/A	N/A
Note 3	t(2)=3.790 PC=56.99	t(2)=-1.625 PC=-4.58	t(2)=-2.173 PC=-109.41	t(2)=.972 PC=34.80	**t(2)=7.210*** **PC=11.16**	**t(2)=4.456*** **PC=7.30**	N/A	N/A
Note 4	**t(2)=8.200*** **PC=78.35**	N/A	N/A	N/A	N/A	t(2)=.386 PC=6.40	t(2)=-.052 PC=-1.28	**t(2)=4.947*** **PC=20.05**
Note 5	t(2)=1.871 PC=28.13	t(2)=1.731 PC=2.20	t(2)=-.979 PC=-50.54	t(2)=.639 PC=21.59	t(2)=.602 PC=11.06	t(2)=.742 PC=1.66	N/A	N/A

Table A- 4 Bird lB.Bl: Call 17

	Duration	Start Frequency	End Frequency	Top Frequency	Peak Frequency	F_{max}	f_0	NPF
Total call	**t(9)=12.267** **PC=39.55**							
Note 1	**t(9)=3.413*** **PC=40.22**	t(9)=-1.157 PC=-7.76	**t(9)=-14.761**** **PC=-49.85**	t(9)=-.594 PC=-2.77	**t(9)=-2.651*** **PC=-10.71**	**t(9)=-3.338*** **PC=-12.01**	N/A	N/A
Note 2	t(9)=-2.067 PC=-12.28	t(9)=-.544 PC=-2.10	t(9)=-5.201 PC=-14.06	N/A	**t(9)=3.531*** **PC=2.58**	**t(9)=3.009*** **PC=2.01**	N/A	N/A
Note 3	**t(9)=3.674*** **PC=17.97**	t(9)=-1.350 PC=-18.59	**t(9)=-5.593**** **PC=-126.64**	t(9)=.652 PC=11.26	t(9)=1.508 PC=11.72	**t(9)=6.062**** **PC=7.62**	N/A	N/A
Note 4	t(9)=1.989 PC=50.38	N/A	N/A	N/A	N/A	**t(9)=-2.788*** **PC=-73.82**	t(9)=-.486 PC=-20.46	**t(9)=-10.309**** **PC=-76.68**
Note 5	**No notes post-lesion**	**No notes post-lesion**	**No notes post-lesion**	N/A	**No notes post-lesion**	**No notes post-lesion**	N/A	N/A
Note 6	**No notes post-lesion**	**No notes post-lesion**	**No notes post-lesion**	**No notes post-lesion**	**No notes post-lesion**	**No notes post-lesion**	N/A	N/A
Note 7	**No notes post-lesion**	N/A	N/A	N/A	N/A	**No notes post-lesion**	**No notes post-lesion**	**No notes post-lesion**

Bird WhWh.OO HVC lesion (hit/hit lesion)

Table A- 5 Bird WhWh.OO: Call 73

	Duration	Start Frequency	End Frequency	Top Frequency	Peak Frequency	F_{max}	f_0	NPF
Total call	**t(18)=6.986** PC=21.16**							
Note 1	**No notes post**	**No notes post-lesion**	**No notes post-lesion**	**No notes post-lesion**	**No notes post-lesion**	**No notes post-lesion**	N/A	N/A
Note 2	t(18)=-1.268 PC=-8.82	t(18)=.490 PC=0.91	**t(18)=3.977* PC=3.71**	N/A	t(18)=1.381 PC=6.75	t(18)=1.775 PC=13.61	N/A	N/A
Note 3	t(18)=.528 PC=1.82	t(18)=.146 PC=0.60	t(18)=1.474 PC=6.42	N/A	**t(18)=3.416* PC=4.22**	**t(18)=3.359* PC=4.41**	N/A	N/A
Note 4	**t(18)=2.785* PC=13.83**	**t(18)=3.391* PC=4.86**	**t(18)=-3.761* PC=-82.61**	**t(18)=2.339* PC=26.12**	t(18)=1.267 PC=1.63	t(18)=-.103 PC=-0.16	N/A	N/A
Note 5	t(17)=1.233 PC=10.32	N/A	N/A	N/A	N/A	t(17)=-1.363 PC=-15.95	**t(17)=4.222* PC=22.32**	**t(17)=-2.255* PC=-16.74**

Table A- 6 WhWh.OO: Call 74

	Duration	Start Frequency	End Frequency	Top Frequency	Peak Frequency	F_{max}	f_0	NPF
Total call	t(18)=1.174 PC=1.80							
Note 1	t(18)=-.587 PC=-.42	**t(18)=2.665*** **PC=3.76**	t(18)=.415 PC=2.23	N/A	t(18)=1.065 PC=4.77	t(18)=.746 PC=3.41	N/A	N/A
Note 2	t(18)=1.085 PC=3.65	**t(18)=2.830*** **PC=5.59**	t(18)=-1.268 PC=-5.65	N/A	**t(18)=2.977*** **PC=4.00**	t(18)=-.135 PC=-0.18	N/A	N/A
Note 3	**t(18)=3.559*** **PC=15.66**	t(18)=-.627 PC=-2.39	**t(18)=-17.252**** **PC=-105.87**	**t(18)=6.275**** **PC=40.17**	**t(18)=3.242*** **PC=2.97**	**t(18)=2.162*** **PC=1.72**	N/A	N/A
Note 4	t(18)=.226 PC=1.85	N/A	N/A	N/A	N/A	t(18)=-.007 PC=-0.09	t(18)=1.538 PC=12.12	t(18)=-.388 PC=-2.64
Note 5	t(18)=1.921 PC=8.36	**t(18)=4.311**** **PC=7.67**	t(18)=-.195 PC=-1.00	N/A	**t(18)=2.877*** **PC=6.78**	t(18)=1.270 PC=3.49	N/A	N/A
Note 6	**t(18)=2.767*** **PC=7.81**	**t(18)=3.612*** **PC=4.14**	**t(18)=-19.497**** **PC=-110.70**	**t(18)=3.889*** **PC=29.02**	t(18)=1.784 PC=1.58	**t(18)=2.206*** **PC=1.88**	N/A	N/A
Note 7	**t(18)=4.222*** **PC=36.99**	N/A	N/A	N/A	N/A	t(18)=1.124 PC=9.49	**t(18)=3.828*** **PC=19.42**	t(18)=-.651 PC=-8.33

Table A- 7 WhWh.OO: Call 75

	Duration	Start Frequency	End Frequency	Top Frequency	Bottom Frequency	Mid Frequency	Peak Frequency	F_{max}	f_0	NPF
Total call	t(18)=1.798 PC=7.54									
Note 1	**t(18)=-2.395*** **PC=-17.20**	t(18)=0.994 PC=0.49	t(18)= 2.228 PC=15.00	N/A	N/A	N/A	t(18)= -.991 PC=-4.66	t(18)= -1.361 PC=-8.74	N/A	N/A
Note 2	t(18)=.145 PC=1.12	**t(18)=2.153*** **PC=17.35**	**t(18)=9.090*** **PC=8.53**	t(18)=1.885 PC=10.24	**t(18)=3.585*** **PC=3.07**	N/A	t(18)= 1.128 PC=15.70	t(18)=-.213 PC=-3.45	N/A	N/A
Note 3	t(18)=-.189 PC=-0.90	t(18)=-1.707 PC=-11.83	t(18)=.879 PC=4.15	N/A	N/A	N/A	t(18)= 1.130 PC=1.08	t(18)= 1.337 PC=1.21	N/A	N/A
Note 4	**t(18)=-4.815**** **PC=-6.79**	t(18)=.567 PC=5.43	t(18)=1.743 PC=5.64	**t(18)=4.583*** **PC=28.96**	t(18)=.920 PC=-12.21	N/A	**t(18)=7.207**** **PC=4.77**	**t(18)=6.132**** **PC=4.54**	N/A	N/A
Note 5	t(18)=1.250 PC=8.90	**t(18)=4.050**** **PC=29.09**	t(18)=-.195 PC=-1.00	N/A	N/A	**t(18)=3.509*** **PC=13.46**	t(18)=-1.187 PC=-9.49	t(18)=-.300 PC=-2.76	N/A	N/A
Note 6	**t(17)=4.531**** **PC=51.29**	N/A	N/A	N/A	N/A	N/A	N/A	**t(17)=-7.258**** **PC=-44.15**	t(18)=1.136 PC=6.92	t(18)=-.545 PC=-6.06

Table A- 8 WhWh.OO: Call 76

	Duration	Start Frequency	End Frequency	Top Frequency	Bottom Frequency	Mid Frequency	Peak Frequency	F_{max}	f_0	NPF
Total call	t(18)=1.684 PC=9.64									
Note 1	t(5)=-.745 PC=-41.24	t(5)=1.712 PC=12.06	t(5)= 1.519 PC=8.86	N/A	N/A	N/A	t(5)= 1.495 PC=8.93	t(5)= 1.373 PC=8.51	N/A	N/A
Note 2	**t(18)=-3.411*** **PC=-7.62**	t(18)=1.100 PC=2.25	t(18)=.388 PC=0.63	**t(17)=3.023*** **PC=2.20**	N/A	N/A	t(18)=.620 PC=2.33	t(18)=.909 PC=3.19	N/A	N/A
Note 3	**t(18)=-4.352**** **PC=-6.04**	t(18)=-1.365 PC=-11.42	**t(18)=6.306**** **PC=6.21**	**t(18)=5.511**** **PC=34.85**	N/A	N/A	**t(18)= 18.311**** **PC=8.95**	**t(18)= 2.364*** **PC=6.98**	N/A	N/A
Note 4	**t(18)=-3.362*** **PC=-3.25**	**t(18)=4.096*** **PC=12.11**	**t(18)=2.690*** **PC=5.91**	N/A	t(18)=-.993 PC=-12.32	**t(15)=18.109**** **PC=16.86**	t(18)=.632 PC=1.65	**t(18)=-4.702*** **PC=-19.01**	N/A	N/A
Note 5	**t(18)=3.362*** **PC=21.51**	N/A	N/A	N/A	N/A	N/A	N/A	**t(18)=-3.752**** **PC=-6.03**	t(18)=-1.064 PC=-5.99	**t(18)=-4.251*** **PC=-14.32**

Bird RG.lB HVC lesion (hit/hit lesion)

Table A- 9 Bird RG.lB: Call 2

	Duration	Start Frequency	End Frequency	Peak Frequency	F_{max}	f_0	NPF
Total call	t(15)=.341 PC=1.83						
Note 1	t(14)=2.117 PC=8.34	**t(14)=3.070*** **PC=2.51**	t(14)=-.907 PC=-1.46	t(14)=-.436 PC=-1.61	t(14)=-.042 PC=-0.17	N/A	N/A
Note 2	**t(15)=2.698*** **PC=16.01**	**t(15)=13.738*** **PC=15.56**	t(15)=-1.801 PC=-33.27	**t(15)=4.855**** **PC=9.64**	**t(15)=4.838**** **PC=8.83**	N/A	N/A
Note 3	t(15)=-1.336 PC=-2.89	t(15)=-.316 PC=-5.92	t(15)=-.872 PC=-14.49	**t(15)=3.193*** **PC=5.53**	**t(15)=2.827*** **PC=10.40**	N/A	N/A
Note 4	**t(15)=3.656*** **PC=34.88**	**t(15)=-3.490*** **PC=-16.90**	**t(15)=7.106**** **PC=13.34**	t(15)=1.310 PC=4.70	t(15)=1.475 PC=6.09	N/A	N/A
Note 5	t(15)=1.662 PC=3.14	N/A	N/A	N/A	t(15)=1.149 PC=7.69	t(15)=-.503 PC=-2.21	t(15)=.208 PC=1.10

Table A- 10 Bird RG.lB Call 3

	Duration	Start Frequency	End Frequency	Peak Frequency	F_{max}	f_0	NPF
Total call	**t(18)=2.597* PC=11.23**						
Note 1	t(16)=-.374 PC=-2.39	**t(16)=3.982* PC=10.43**	**t(16)=3.248* PC=30.08**	**t(16)=13.811** PC=10.12**	**t(16)=15.857** PC=9.71**	N/A	N/A
Note 2	t(18)=-1.139 PC=-15.28	t(18)=.730 PC=0.48	t(18)=1.499 PC=13.01	**t(18)=2.344* PC=6.13**	**t(18)=2.424* PC=9.28**	N/A	N/A
Note 3	t(17)=.379 PC=1.31	t(17)=1.024 PC=11.44	**t(17)=2.527* PC=9.92**	**t(17)=27.357** PC=9.33**	**t(17)=27.134** PC=9.28**	N/A	N/A
Note 4	**t(18)=-2.904* PC=-51.48**	N/A	N/A	N/A	t(18)=.126 PC=0.92	N/A	N/A
Note 5	**t(17)=-2.137* PC=-36.44**	t(17)=1.661 PC=5.31	t(17)=-1.669 PC=-13.47	t(17)=-1.146 PC=-3.20	t(17)=-1.724 PC=-5.32	N/A	N/A
Note 6	t(17)=.822 PC=9.84	N/A	N/A	N/A	**t(17)=2.639* PC=31.40**	**t(17)=5.099** PC=31.20**	**t(17)=3.847* PC=25.72**

Table A- 11 Bird RG.lB: Call 4

	Duration	Start Frequency	End Frequency	Peak Frequency	F_{max}	f_0	NPF
Total call	t(15)=2.173 PC=14.23						
Note 1	**t(15)=2.990*** **PC=11.93**	t(15)=.373 PC=1.85	t(15)=-1.676 PC=-23.13	N/A	**t(15)=4.116*** **PC=7.87**	t(15)=.328 PC=1.18	N/A
Note 2	t(15)=-.676 PC=-3.11	t(15)=1.557 PC=6.50	t(15)=.026 PC=0.26	N/A	**t(15)=4.908*** **PC=4.95**	**t(15)=3.532*** **PC=4.63**	N/A
Note 3	t(15)=1.205 PC=13.38	t(15)=-2.293 PC=-16.89	**t(15)=4.274*** **PC=12.21**	N/A	t(15)=.627 PC=2.69	t(15)=.150 PC=1.63	N/A
Note 4	**t(15)=3.500*** **PC=33.70**	N/A	N/A	t(15)=1.205 PC=7.35	N/A	t(15)=-2.033 PC=-49.83	t(15)=-1.404 PC=-9.47

Table A- 12 Bird RG.lB: Call 5

	Duration	Start Frequency	End Frequency	Top Frequency	Peak Frequency	Fmax	F0	NPF
Total call	t(12)=2.293 PC=31.64							
Note 1	t(12)=-1.127 PC=-27.20	t(12)=-1.409 PC=-5.27	t(12)=1.400 PC=2.48	N/A	**t(12)=2.395* PC=5.30**	t(12)=-1.740 PC=-10.04	N/A	N/A
Note 2	t(12)=1.726 PC=17.03	t(12)=2.020 Pc=19.37	**t(12)=6.457** PC=12.76**	**t(12)=5.365** PC=7.17**	**t(12)=7.852** PC=51.56**	t(12)=1.833 PC=25.62	N/A	N/A
Note 3	**t(12)=2.344* PC=3.94**	t(12)=1.872 PC=6.86	**t(12)=3.836* PC=9.34**	t(12)=-.809 PC=-13.67	t(12)=.214 PC=1.27	**t(12)=9.147** PC=6.60**	N/A	N/A
Note 4	t(12)=.916 PC=14.89	t(12)=-1.763 PC=-7.52	t(12)=1.945 PC=14.90	N/A	t(12)=.476 PC=1.05	t(12)=-.305 PC=-0.82	N/A	N/A
Note 5	t(12)=1.257 Pc=27.90	N/A	N/A	N/A	N/A	**t(12)=3.734* PC=34.20**	t(12)=1.337 PC=13.79	**t(12)=3.713* PC=26.77**
Note 6	**t(9)=-4.411* PC=-8.17**	t(9)=1.411 PC=8.21	t(9)=.989 PC=27.03	t(9)=.191 PC=4.35	**t(9)=2.332* PC=6.31**	**t(9)=4.958* PC=7.78**	N/A	N/A
Note 7	t(9)=.471 PC=8.82	t(9)=.997 PC=8.43	t(9)=2.066 PC=10.73	N/A	t(9)=1.307 PC=7.06	t(9)=.781 PC=6.113	N/A	N/A
Note 8	t(9)=-.274 PC=-1.53	N/A	N/A	N/A	N/A	t(9)=.959 PC=37.84	t(9)=1.502 PC=16.13	t(9)=1.878 PC=37.20

Bird GrPe.O HVC lesion (hit/hit lesion)

Table A- 13 Bird GrPe.O: Call 1

	Duration	Start Frequency	End Frequency	Peak Frequency	Minimum Frequency	Fmax	F0	NPF
Total call	t(15)=-1.238 PC=-4.96							
Note 1	t(15)=.253 PC=1.22	t(15)=1.894 PC=1.85	t(15)=-.783 PC=-2.30	t(15)=1.509 PC=0.95	N/A	t(15)=1.510 PC=0.85	N/A	N/A
Note 2	t(15)=-.139 PC=-0.79	t(15)=1.676 PC=3.56	**t(15)=-2.264* PC=-7.19**	t(15)=1.742 PC=2.49	N/A	**t(15)=-5.324** PC=-17.29**	N/A	N/A
Note 3	t(15)=-1.164 PC=-3.80	t(15)=-.644 PC=-5.26	t(15)=-2.212 PC=-11.29	t(15)=.239 PC=2.52	t(15)=-1.442 PC=-20.77	t(15)=.304 PC=1.98	N/A	N/A
Note 4	t(15)=.870 PC=14.59	N/A	N/A	N/A	N/A	**t(15)=3.960* PC=13.37**	t(15)=1.924 PC=11.71	t(15)=1.819 PC=9.40

Bird Br.O HVC lesion (miss/miss lesion)

Table A- 14 Bird Br.O: Call 97

	Duration	Start Frequency	End Frequency	Peak Frequency	Top Frequency	Fmax	F0	NPF
Total call	t(13)=0.082 PC=0.22							
Note 1	t(13)=0.452 PC=2.36	**t(13)=4.519*** **PC=6.25**	t(13)=1.035 PC=2.02	t(13)=1.541 PC=2.21	N/A	t(13)=1.842 PC=2.49	N/A	N/A
Note 2	t(13)=-.842 PC=-6.30	t(13)=.930 PC=2.73	t(13)=-1.245 PC=-4.79	t(13)=-.046 PC=0.07	N/A	t(13)=1.109 PC=1.12	N/A	N/A
Note 3	t(13)=-1.748 PC=-3.32	t(13)=.143 PC=0.11	t(13)=.901 PC=13.23	t(13)=1.260 PC=6.95	t(13)=-1.55 PC=-9.97	t(13)=1.560 PC=2.47	N/A	N/A
Note 4	t(13)=-.203 PC=-2.71	t(13)=-1.652 PC=0.12	t(13)=1.135 PC=2.39	t(13)=-.957 PC=-2.75	N/A	t(13)=-.632 PC=-1.93	N/A	N/A
Note 5	**t(13)=2.539*** **PC=14.49**	N/A	N/A	N/A	N/A	t(13)=-.629 PC=-9.59	t(13)=1.179 PC=17.13	t(13)=-.891 PC=-5.65

Table A- 15 Bird Br.O: Call 98

	Duration	Start Frequency	End Frequency	Peak Frequency	Top Frequency	Fmax	F0	NPF
Total call	**t(18)=-3.292* PC=-9.80**							
Note 1	t(18)=-.961 PC=-35.20	**t(18)=2.867* PC=3.82**	**t(18)=11.341** PC=5.98**	**t(18)=6.930** PC=5.02**	N/A	**t(18)=7.614** PC=4.80**	N/A	N/A
Note 2	t(18)=1.515 PC=7.12	**t(18)=6.136** PC=5.75**	t(18)=1.318 PC=3.26	**t(18)=7.712** PC=3.70**	N/A	**t(18)=10.799** PC=3.81**	N/A	N/A
Note 3	t(18)=-2.095 PC=-2.92	**t(18)= 6.932** PC=3.29**	t(18)=-.691 PC=-3.87	t(18)=1.84 PC=6.96	t(18)=1.856 PC=10.18	**t(18)=5.587** PC=3.26**	N/A	N/A
Note 4	t(18)=1.199 PC=10.75	t(18)=-1.239 PC=-2.72	t(18)=1.509 PC=1.83	t(18)=.765 PC=2.58	N/A	t(18)=.396 PC=1.52	N/A	N/A
Note 5	t(18)=.679 PC=2.08	N/A	N/A	N/A	N/A	t(18)=-1.994 PC=-13.03	**t(18)=-2.804* PC=-19.03**	**t(18)=-2.911* PC=-10.80**
Note 6	t(18)=.217 PC=1.42	**t(18)=2.667* PC=12.40**	t(18)=1.021 PC=3.29	**t(18)=2.597* PC=1.76**	N/A	**t(18)=3.742* PC=2.33**	N/A	N/A
Note 7	t(18)=-1.412 PC=-2.28	**t(18)=5.078** PC=4.02**	t(18)=.358 PC=1.61	t(18)=1.282 PC=7.45	**t(18)=2.116* PC=12.04**	t(18)=1.300 PC=5.19	N/A	N/A
Note 8	t(18)=.722 PC=6.49	t(18)=.313 PC=0.61	t(18)=.703 PC=1.96	t(18)=.447 PC=1.47	N/A	t(18)=.594 PC=2.18	N/A	N/A
Note 9	**t(18)=-2.331* PC=-42.35**	N/A	N/A	N/A	N/A	t(18)=-1.242 PC=-5.21	**t(18)=-2.558* PC=-15.99**	t(18)=.455 PC=3.75

Bird BGr.Y HVC lesion (miss/miss lesion)

Table A- 16 BGr.Y: Call 88

	Duration	Ascending Duration	Descending Duration	Start Frequency	End Frequency	Peak Frequency	Fmax	F0	NPF
Total call	**t(15)=-11.260** **PC=-30.85**								
Note 1 Duration	**t(15)=6.252** **PC=32.35**	N/A	N/A	**t(15)=6.975** **PC=6.28**	**t(15)=-67.426** **PC=-80.80**	t(15)=.270 PC=0.25	t(15)=-.260 PC=-0.26	N/A	N/A
Note 2 Duration	t(15)=2.132 PC=12.57	N/A	N/A	t(15)=-1.488 PC=-4.75	**t(15)=7.640** **PC=39.09**	t(15)=-.613 PC=-0.36	t(15)=-1.743 PC=-0.82	N/A	N/A
Note 3 Duration	**t(15)=3.999*** **PC=14.73**	**t(15)=-5.237**** **PC=-73.48**	t(15)=-.380 PC=-4.51	t(15)=-.078 PC=-0.56	**t(15)=-2.336*** **PC=-18.81**	**t(15)=-12.196**** **PC=-7.54**	**t(15)=-10.554**** **PC=-8.08**	N/A	N/A
Note 4 Duration	**t(15)=-5.318**** **PC=-69.13**	N/A	N/A	t(15)=1.170 PC=3.35	t(15)=-1.508 PC=-8.57	t(15)=-.430 PC=-1.86	t(15)=1.138 PC=9.56	N/A	N/A
Note 5 Duration	**t(15)=-6.463**** **PC=-122.75**	N/A	N/A	N/A	N/A	N/A	**t(15)=11.929**** **PC=45.95**	**t(15)=9.785**** **PC=39.79**	**t(15)=2.169*** **PC=9.22**

Appendix B: Effects of HVC lesions on *gargle* calls. T-tests are provided for pre- and post-lesion comparisons of bioacoustic measurements. PC = percent change in the parameter, provided as an estimate of effect size of the lesion.

Table B- 1 Bird lB.Bl HVC lesion (hit/hit lesion)

	Duration	Ascending Duration	Descending Duration	Start Frequency	End Frequency	Peak Frequency	Fmax	F0	NPF
Total call	**t(12)=2.950* PC=41.41**								
A note	t(11)=.828 PC=6.92	t(11)-.815 PC=-8.80	t(11)=.553 PC=9.97	**t(11)=2.825* PC=18.57**	t(11)=.947 PC=5.98	**t(11)=3.466* PC=8.14**	**t(11)=3.336* PC=8.19**	N/A	N/A
B note	**t(23)=-4.625** PC=-33.37**	**t(23)=-2.235* PC=-20.28**	t(23)=-.576 PC=-12.46	t(23)=.770 PC=5.42	t(23)=-1.104 PC=-9.78	t(23)=1.945 PC=7.65	t(23)=1.977 PC=8.00	N/A	N/A
D note	t(38)=.937 PC=16.79	N/A	N/A	N/A	N/A	N/A	t(38)=.174 PC=1.16	t(38)=-1.617 PC=-32.34	t(38)=-.564 PC=-10.40

Table B- 2 Bird WhWh.OO HVC lesion (hit/hit lesion)

	Duration	Ascending Duration	Descending Duration	Start Frequency	End Frequency	Peak Frequency	Fmax	F0	NPF
Total call	**t(18)=2.863* PC=35.90**								
A note	**t(36)=-4.349* PC=33.22**	t(36)=-.864 PC=-4.43	**t(36)=-6.384** PC=-122.93**	**t(36)=4.540** PC=12.53**	**t(36)=3.463* PC=20.93**	**t(36)=7.431** PC=10.27**	**t(36)=6.318** PC=9.12**	N/A	N/A
B note	t(7)=-1.651 PC=-61.06	**t(7)=-3.577* PC=-35.90**	t(7)=-1.589 PC=-115.34	t(7)=-.837 PC=-6.02	t(7)=-.539 PC=-7.35	t(7)=-.015 PC=-0.12	t(7)=.094 PC=0.74	N/A	N/A
C note	No notes post-lesion	No notes post-lesion	No notes post-lesion	No notes post-lesion	No notes post-lesion	No notes post-lesion	No notes post-lesion	N/A	N/A
D note	**t(34)=-3.090 PC=-14.06**	N/A	N/A	N/A	N/A	N/A	t(34)=-.275 PC=-0.69	**t(34)=-3.946** PC=-19.82**	**t(34)=3.422* PC=19.68**

Table B- 3 Bird RG.lB HVC lesion (hit/hit lesion)

	Duration	Ascending Duration	Descending Duration	Start Frequency	End Frequency	Peak Frequency	Fmax	F0	NPF
Total call	**t(18)=3.418* PC=50.62**								
A note	t(8)=.431 PC=3.43	t(8)=1.097 PC=12.96	t(8)=.802 PC=8.17	t(8)=-.890 PC=-2.83	t(8)=-2.072 PC=-16.67	t(8)=-.793 PC=-2.18	t(8)=-.647 PC=-1.42	N/A	N/A
B note	t(19)=.023 PC=0.16	t(19)=.313 PC=1.96	t(19)=.057 PC=0.70	t(19)=-.075 PC=-0.40	t(19)=.957 PC=12.01	t(19)=-.557 PC=-1.29	t(19)=-.683 PC=-1.60	N/A	N/A
C note	No notes post-lesion	No notes post-lesion	No notes post-lesion	No notes post-lesion	No notes post-lesion	No notes post-lesion	No notes post-lesion	N/A	N/A
D note	**t(45)=6.391** PC=19.74**	N/A	N/A	N/A	N/A	N/A	t(45)=1.733 PC=15.71	t(45)=.320 PC=1.52	**t(45)=4.115** PC=26.72**

Table B- 4 Bird GrPe.O HVC lesion (hit/hit lesion)

	Duration	Ascending Duration	Descending Duration	Start Frequency	End Frequency	Peak Frequency	Fmax	F0	NPF
Total call	**t(18)=-2.431* PC=-52.38**								
A note	t(23)=.605 PC=3.77	**t(23)=-3.324* PC=-66.62**	t(23)=-.177 PC=-3.31	t(23)=.251 PC=0.75	t(23)=.980 PC=6.58	t(23)=-1.651 PC=-2.43	t(23)=-1.169 PC=-1.73	N/A	N/A
B note	t(15)=.758 PC=5.75	t(15)=-.028 PC=-1.18	t(15)=1.410 PC=20.15	**t(15)=3.196* PC=27.87**	**t(15)=3.521* PC=25.62**	t(15)=-.226 PC=-2.03	t(15)=-.228 PC=-2.03	N/A	N/A
C note	No notes post-lesion	No notes post-lesion	No notes post-lesion	No notes post-lesion	No notes post-lesion	No notes post-lesion	No notes post-lesion	N/A	N/A
D note	**t(59)=-4.048** PC=-9.89**	N/A	N/A	N/A	N/A	N/A	t(59)=1.254 PC=3.82	t(59)=-.632 PC=-4.35	t(59)=.154 PC=0.76

Table B- 5 Bird Br.O Missed lesion (miss/miss lesion)

	Duration	Ascending Duration	Descending Duration	Start Frequency	End Frequency	Peak Frequency	Fmax	F0	NPF
Total call	**t(12)=-2.553* PC=-65.76**								
A note	t(15)=1.356 PC=12.89	t(15)=1.238 PC=14.37	t(15)=-.959 PC=-18.38	**t(15)=3.343* PC=15.44**	**t(15)=3.228* PC=30.15**	t(15)=1.726 PC=6.56	t(15)=1.655 PC=6.10	N/A	N/A
B note	t(13)=.258 PC=2.76	t(13)=1.600 PC=20.42	t(13)=.065 PC=0.44	**t(13)=2.403* PC=15.24**	t(13)=-.173 PC=-2.52	**t(13)=3.177* PC=14.92**	**t(13)=3.317* PC=15.39**	N/A	N/A
D note	t(25)=-1.210 PC=-8.56	N/A	N/A	N/A	N/A	N/A	t(25)=.709 PC=1.16	t(25)=-.463 PC=-4.89	t(25)=-.483 PC=-4.01

Table B- 6 Bird BGr.Y Missed lesion (miss/miss lesion)

	Duration	Ascending Duration	Descending Duration	Start Frequency	End Frequency	Peak Frequency	Fmax	F0	NPF
Total call	**t(18)=-3.038* PC=-43.06**								
A note	t(25)=1.208 PC=8.20	**t(25)=-3.269* PC=-36.53**	**t(25)=3.071* PC=27.20**	t(25)=1.767 PC=6.37	t(25)=-1.877 PC=-9.54	t(25)=1.926 PC=5.49	t(25)=1.801 PC=4.67	N/A	N/A
B note	t(16)=-.990 PC=-14.15	t(16)=-.912 PC=-12.13	t(16)=-.862 PC=-17.83	t(16)=-.354 PC=-2.07	t(16)=-.940 PC=-8.87	t(16)=.548 PC=2.03	t(16)=1.380 PC=5.54	N/A	N/A
D note	**t(63)=-2.271* PC=-3.12**	N/A	N/A	N/A	N/A	N/A	t(63)=-1.309 PC=-3.59	t(63)=1.278 PC=4.12	t(63)=1.192 PC=4.51

Appendix C: Animal use protocol

From: **eSiriusWebServer** esiriusadmin@uwo.ca
Subject: **eSirius Notification - New Animal Use Protocol is APPROVED2015-043::1**
Date: October 20, 2015 at 15:10
To: smacdou2@uwo.ca
Cc: auspc@uwo.ca, esiriusadmin@uwo.ca

Western

AUP Number: 2015-043
PI Name: Macdougallshackleton, Scott A
AUP Title: Neurobiology Of Songbird Vocal Learning
Approval Date: 10/20/2015

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Neurobiology Of Songbird Vocal Learning" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2015-043::1

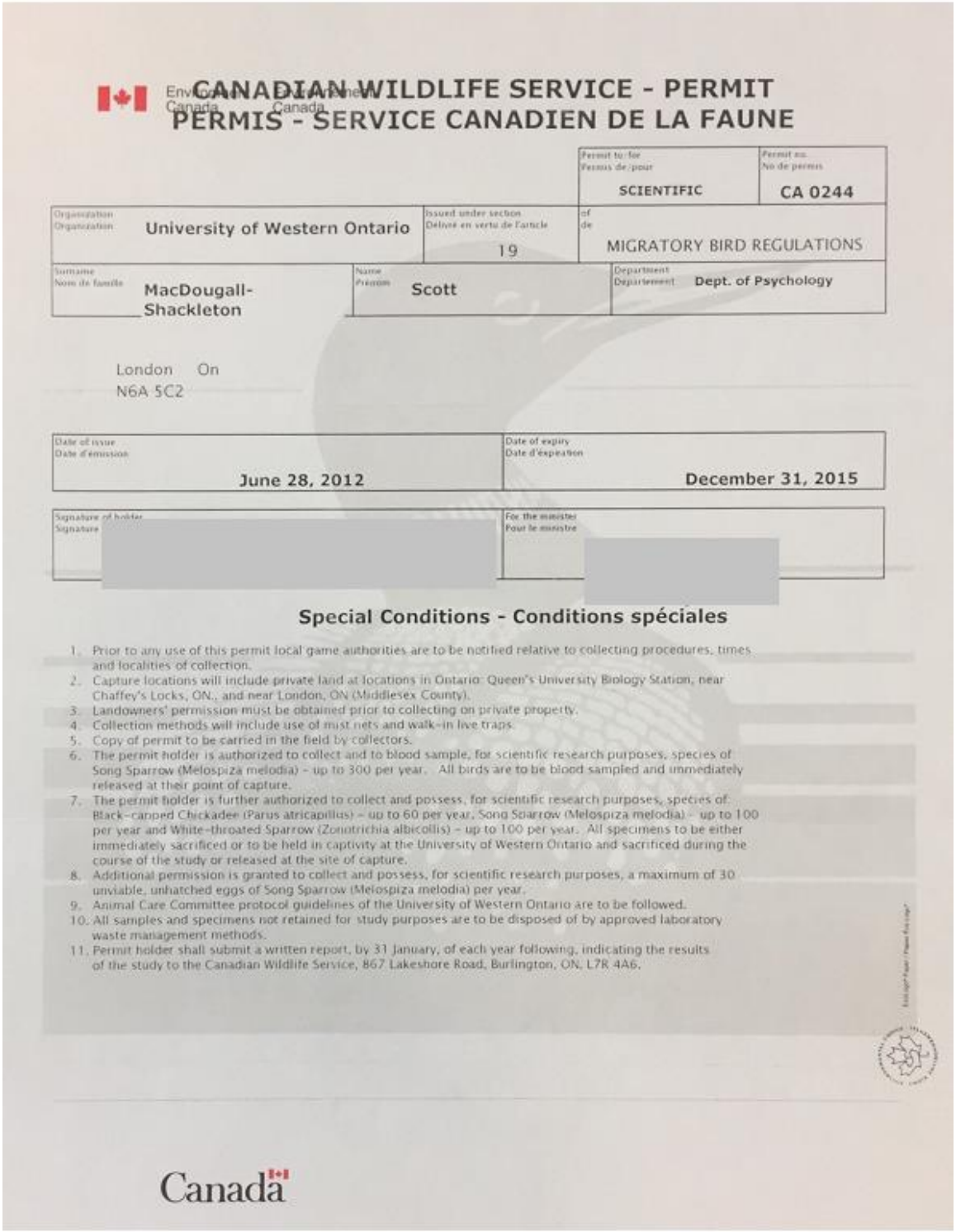
1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

The University *of* Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, ● London, Ontario ● CANADA – N6A 5C1
PH: 519-661-2111 ext. 86768 ● FL 519-661-2028
Email: auspc@uwo.ca ● http://www.uwo.ca/animal/website/

Appendix D: Canadian wildlife service permit



Environment Canada / Environnement Canada

CANADIAN WILDLIFE SERVICE - PERMIT
PERMIS - SERVICE CANADIEN DE LA FAUNE

Permit to / for Permis de / pour	Permit no. No de permis
SCIENTIFIC	CA 0244

Organization Organization	Issued under section Délivré en vertu de l'article	of de
University of Western Ontario	19	MIGRATORY BIRD REGULATIONS

Surname Nom de famille	Name Prénom	Department Département
MacDougall-Shackleton	Scott	Dept. of Psychology

London On
N6A 5C2

Date of issue Date d'émission	Date of expiry Date d'expiration
June 28, 2012	December 31, 2015

Signature of holder Signature	For the minister Pour le ministre
[redacted]	[redacted]

Special Conditions - Conditions spéciales

1. Prior to any use of this permit local game authorities are to be notified relative to collecting procedures, times and localities of collection.
2. Capture locations will include private land at locations in Ontario: Queen's University Biology Station, near Chaffey's Locks, ON., and near London, ON (Middlesex County).
3. Landowners' permission must be obtained prior to collecting on private property.
4. Collection methods will include use of mist nets and walk-in live traps.
5. Copy of permit to be carried in the field by collectors.
6. The permit holder is authorized to collect and to blood sample, for scientific research purposes, species of Song Sparrow (Melospiza melodia) - up to 300 per year. All birds are to be blood sampled and immediately released at their point of capture.
7. The permit holder is further authorized to collect and possess, for scientific research purposes, species of: Black-capped Chickadee (Parus atricapillus) - up to 60 per year, Song Sparrow (Melospiza melodia) - up to 100 per year and White-throated Sparrow (Zonotrichia albicollis) - up to 100 per year. All specimens to be either immediately sacrificed or to be held in captivity at the University of Western Ontario and sacrificed during the course of the study or released at the site of capture.
8. Additional permission is granted to collect and possess, for scientific research purposes, a maximum of 30 unviable, unhatched eggs of Song Sparrow (Melospiza melodia) per year.
9. Animal Care Committee protocol guidelines of the University of Western Ontario are to be followed.
10. All samples and specimens not retained for study purposes are to be disposed of by approved laboratory waste management methods.
11. Permit holder shall submit a written report, by 31 January, of each year following, indicating the results of the study to the Canadian Wildlife Service, 867 Lakeshore Road, Burlington, ON, L7R 4A6.

Canada

Curriculum Vitae

Shannon K. Mischler

POSITIONS

Post-doctoral fellow, University of Alberta (2017-present)

- Examining the role of urban noise on call discrimination and neurogenesis in hippocampus and the vocal control system, and following up on HVC lesion results, by examining if black-capped chickadees have a neural basis of perception difference for intact vs. lesioned *gargle* calls. Also investigating the role of HVC in the neural perception of the *gargle*, chick-a-*dee* and *fee-bee* song in CMM and NCM.

EDUCATION

PhD., Psychology, University of Western Ontario (2012-2017)

- Current thesis: The neural mechanisms underlying the perception and production of learned vocalizations in songbirds with Dr. Scott MacDougall-Shackleton

MSc., Psychology, Wilfrid Laurier University (2010-2012)

- Thesis: Many-to-one matching with temporal and hedonic samples in rats with Dr. Angelo Santi

HBSc., Psychology and Biology, University of Toronto (2004-2008)

SCHOLARSHIPS AND AWARDS

2016

- 3 minute thesis competition top 20 finalist at the University of Western Ontario, broadcasted on Rogers TV, https://www.youtube.com/watch?v=jHbULsrHTMQ&t=3s
- Graduate Research Scholarship (10900$), University of Western Ontario

2015

- Oral Presentation Award (2nd place, 100$) at the Ontario Ecology, Ethology, and Evolution Colloquium
- Graduate Research Scholarship (10900$), University of Western Ontario

2014

- Graduate Research Scholarship (10900$), University of Western Ontario

2013

- Graduate Research Scholarship (10900$), University of Western Ontario

2012

- Graduate Scholarship (1000$), Wilfrid Laurier University
- General Graduate Bursary (1950$), Wilfrid Laurier University

2011

- Graduate Scholarship (1667$), Wilfrid Laurier University
- General Graduate Bursary (2400$), Wilfrid Laurier University

2010

- Graduate Scholarship (333$), Wilfrid Laurier University

PUBLICATIONS

Peer-reviewed journal articles

1. Santi, S., Simmons, S., **Mischler, S. K.**, & Hoover, C. (2013). Rats exhibit asymmetrical retention functions for hedonic and nonhedonic samples in many-to-one symbolic delayed matching-to-sample. Learning & Behavior, 41 (2), 168-178. doi: 10.3758/s13420-012-0094-2 (during Master's).
2. Santi, A., Simmons, S., & **Mischler, S**. K. (2011). Rats exhibit asymmetrical retention functions for hedonic samples. Behavioural Processes, 88, 121-126. doi: 10.1016/j.beproc.2011.08.007

Journal articles in preparation

1. Mischler, S. K., Karlin, E. J., & MacDougall-Shackleton (in prep). HVC and RA are active during production of learned aggressive calls in black-capped chickadees (Poecile atricapillus).
2. Mischler, S. K., & MacDougall-Shackleton (in prep). HVC lesions affect the production of the gargle call in black-capped chickadees (Poecile atricapillus).
3. Mischler, S. K., & MacDougall-Shackleton (in prep). The differential response of the auditory regions in response to song and calls in female and male black-capped chickadees (Poecile atricapillus).
4. Mischler, S. K., Sekler, O. Y. & MacDougall-Shackleton (in prep). The effect of HVC lesions on the perception of the long-call in the auditory regions of the zebra finch (Taeniopygia guttata).

Non-refereed Publications

1. Mischler, S. K., Congdon, J. V., Scully, E. N., Campbell, K. A., & Sturdy, C. B. (2017). (In press, accepted June 27th, 2017). Passerine vocal communication. In J.

Vonk & T. Shakelford (Eds), Encyclopedia of Animal Cognition and Behavior. Springer.

Conference Papers

1. Santi, A., Simmons, S. & **Mischler, S. K** (2011, November). Asymmetric retention functions for hedonic samples in rats: The effect of differential head entry behavior. Paper accepted for presentation at the fall meeting of the Comparative Cognition Society, Seattle, Washington.
2. Santi, A, & Simmons, S., **Mischler, S. K**, & Hoover, C. (2011, March). Rats exhibit asymmetrical retention functions for hedonic and nonhedonic samples in many-to-one symbolic delayed matching-to-sample. Paper presented at the International Conference on Comparative Cognition, Melbourne, Florida, USA.

PRESENTATIONS

Conference Presentations and Posters

1. **Mischler, S. K**., & MacDougall-Shackleton, S. A. (2017, April). Differential effects of song and calls on ZENK immediate early gene expression in the auditory forebrain regions of the black-capped chickadee (*Poecile atricapillus*). Talk presented at the 24th International Conference on Comparitive Cognition, Melbourne, Florida, USA.
2. **Mischler, S. K**., & MacDougall-Shackleton, S. A. (2016, May). Song control or vocal control? The role of HVC in black-capped chickadee learned call production. Talk presented at the 55th annual meeting for the Canadian Society of Zoologists, London, ON.
3. **Mischler, S. K**., & MacDougall-Shackleton, S. A. (2016, April). Song control or vocal control? The role of HVC in black-capped chickadee call production. Talk presented at the International Conference on Comparative Cognition, Melbourne, Florida, USA.
4. **Mischler, S. K**., Karlin E. J., & MacDougall-Shackleton, S. A. (2015, May). Song control or vocal control system? The neural basis of learned call production in songbirds. Talk presented at the Ontario Ecology, Ethology and Evolution Colloquium, Toronto, ON.
5. **Mischler, S. K**., Karlin, E. J., & MacDougall-Shackleton, S. A. (2015, May). Song control or vocal control system? The neural basis of learned call production in songbirds. Poster presented at the Southern Ontario Neuroscience Association, Hamilton, ON.
6. **Mischler, S. K., Karlin**, E. J., & MacDougall-Shackleton, S. A. (2014, November). Song control system or vocal-control system? HVC is active during production of learned aggressive calls. Poster presented at the Society for Neuroscience, Washington, DC, USA.

7. **Mischler, S. K**., & MacDougall-Shackleton, S. A. (2014, May). Is the HVC of black-capped chickadees (*Poecile atricapillus*) a vocal-control brain region rather than a song-control brain region?. Talk presented at the Ontario Ecology Ethology and Evolution Colloquium, Guelph, ON.
8. **Mischler, S. K**., & MacDougall-Shackleton, S. A. (2014, May). HVC is activated by the production of the gargle call in black-capped chickadees (Poecile atricapillus). Poster presented at the Southern Ontario Neuroscience Association, London, ON.

9. **Mischler, S. K.**, & MacDougall-Shackleton, S. A. (2013, May). ZENK expression in the song-control system for song and learned calls in the black-capped chickadee (*Poecile atricapillus*). Talk presented at the Ontario Ecology Ethology and Evolution Colloquium, London, ON.
10. **Mischler, S. K.,** & Santi, A. (2012, April). Retention functions for temporal and hedonic samples in many-to-one symbolic delayed matching-to-sample in rats. Poster presented at the Southern Ontario Neuroscience Association, Toronto, ON.
11. **Mischler, S. K.,** & Santi, A. (2012, April). Retention functions for temporal and hedonic samples in many-to-one symbolic delayed matching-to-sample in rats. Poster presented at the annual Neuroscience Research Day at the University of Guelph, Guelph. ON.
12. **Mischler, S. K.** (2012, March). Retention functions for temporal and hedonic samples in many-to-one symbolic delayed matching-to-sample in rats. Poster presented at the International Conference on Comparative Cognition, Melbourne, Florida, USA.
13. Santi, A., Simmons, S. & **Mischler, S.** (2011, November). Asymmetric retention functions for hedonic samples in rats: The effect of within-session and between-session variation in retention interval. Poster accepted for presentation at the meeting of the Psychonomic Society, Seattle, Washington, USA
14. **Mischler, S. K.**, & Santi, A. (2011, May). Many-to-one matching with temporal and hedonic samples in rats. Poster presented at the annual meeting of the Southern Ontario Neuroscience Association, Guelph, ON.

TEACHING EXPERIENCE

Course instructor for the following course at the University of Alberta:

- Brain and behaviour (PSYCO 275), summer 2018
- Evolutionary theory psychology (PSYCO 491), winter 2018

Course instructor for the following course at the University of Western Ontario:

- Evolution and Human Behaviour (PS3229), summer 2016

Teaching assistant for the following courses the University of Western Ontario:

- PS3226: Hormones and Behaviour, winter 2016

- PS3229: Evolution and Human Behaviour, fall 2015
- PS3285: Research in Behavioural Neuroscience, fall and winter 2014-2015
- PS2800 (Lab): Research Methods in Psychology, fall and winter 2013-2014, fall 2012
- PS2800 (Marking): Research Methods in Psychology, winter 2013

Teaching assistant for the following courses at Wilfrid Laurier University:

- PS102: Introduction to Psychology II, winter 2012
- PS101: Introduction to Psychology I, winter 2012
- PS361: Research in Learning, winter 2011
- PS296: Introduction to Statistics, winter 2011
- PS361: Research in Learning, fall 2010

ADMINISTRATIVE ACTIVITY AND COMMUNITY INVOLVEMENT

Founder and Organizer of the AFAR (Advanced Facility for Avian Research) Journal Club (January 2014, September 2016).

- Organized papers for weekly discussions on avian research

Graduate Student Senator at the University of Western Ontario

- In charge of bringing social science student views and issues to the University senate
- Subcommittee: Operations and Agenda
- Subcommittee: Student Review Board Academic

Graduate Student Senator at the University of Western Ontario

- In charge of bringing social science student views and issues to the University senate
- Subcommittee: Honorary Degrees Committee

Presenter at Teaching and Professional Development Conference: Panel for "T.A. ing labs"

- Presented on the topic "what to expect when T.A. ing labs in the sciences", and answered first year graduate students questions.

Ethics review committee member at Wilfrid Laurier University

- Reviewed undergraduate psychology ethics applications for undergraduate thesis students.

WORK EXPERIENCE

Research assistant at Wilfrid Laurier University (2010-2012)

- Conducting experiments in the realm of comparative cognition, including work with pigeons and rats, specifically examining retention functions of rats for hedonic samples with Dr. Angelo Santi.

Research assistant at the University of Toronto (Mississauga) (2007-2008)

- Conducting experiment in the realm of maternal rearing and artificial rearing, specifically examining levels of impulsivity in maternally reared, and artificially reared rats. Also aiding in the collection of data examining pain tolerance in artificially vs. maternally reared rats with Dr. Alison Fleming.