Aspects of Singing Behaviour and Song perception in Two North American Forest Songbirds, Black-capped Chickadee (*Poecile atricapillus)* and Hermit Thrush (*Catharus guttatus*)

by

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Submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
January 2016

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ABSTRACT

Birdsong, used mainly by male songbirds, is centrally important to breeding behaviour as part of both territorial defense and mate attraction. Song perception is therefore important for both males and females, as they rely on song for conspecific recognition (identifying a singer as belonging to the same species) and individual recognition. Songbird species exhibit incredible variety not only with respect to song production, but also in terms of the acoustic features used for conspecific and individual recognition. Reflecting that variety, this thesis examined aspects of song production and conspecific recognition in two songbird species that differ greatly in terms of singing behaviour: the black-capped chickadee (*Poecile atricapillus*), a well-studied species with a single, simple song (*fee bee*), and the hermit thrush (*Catharus guttatus*), a less studied species in which individuals sing multiple versions of songs that are more structurally complex than those of the chickadee.

Previous behavioural work has shown that, for conspecific recognition, female chickadees attend to two relative pitch ratios within the *fee bee* song: the frequency ratio from the start to the end of the *fee* (the *glissando*) and the frequency ratio from the end of the *fee* to the start of the *bee* (the inter-note interval). In contrast, fieldwork suggests that males attend only to the *glissando.* In the first study, I measured the neural response of males and females to songs with those pitch ratios either intact or altered. The lack of differences in brain activity within NCM and CMM, auditory regions associated with discrimination of conspecific versus heterospecific vocalizations, with respect to playback type suggests that discrimination of such relatively subtle structural differences takes place elsewhere in the auditory system. Birds vocalized more to songs with the species-typical inter-note interval than to songs without it, and, in the operant study that followed, birds learned that discrimination with ease. In contrast, birds had difficulty learning to discriminate the presence or absence of the *glissando*, and their vocal responses did not differ when it was altered. This suggests, contrary to previous field work, that the glissando may not be necessary for conspecific recognition.

After an initial study to provide basic bioacoustics analyses of repertoire size, song structure, and song syntax in the little-studied hermit thrush, I examined geographic variation in song structure across the species' large range. My studies describe extensive differences in frequency and temporal song characteristics, both between and within subspecies. I explored the implications of these differences in terms of conspecific recognition using a playback study where territorial males were exposed to local and foreign songs. Males exhibited a stronger territorial response to local songs, suggesting that they can discriminate local from foreign songs. These results are discussed in terms of recent findings that show extensive genetic divergence in the hermit thrush, suggestive of speciation. Taken together, these studies demonstrate the importance of acoustic features to conspecific recognition in two complimentary North American songbird species.

LIST OF ABBREVIATIONS USED

ANOVA	analysis of variance
AST	Atlantic Standard Time
CMM	caudomedial mesopallium
dB	decibels
DFA	discriminant function analysis
DR	discrimination ratio
FF	flat fee
FFFI	flat fee/flat interval
FI	flat interval
FoxP2	Forkhead box protein P2
GG	Greenhouse-Geisser
HFX	Halifax
Hz	hertz
ITI	intertrial interval
kHz	kilohertz
LMAN	lateral magnocellular nucleus of the nidopallium
m	meters
MANOVA	multivariate analysis of variance
ME	Maine
N	Northern
NCM	caudomedial nidopallium
NCMd	dorsal caudomedial nidopallium
NCMv	ventral caudomedial nidopallium
PBS	phosphate-buffered saline
PC	principal component
PCA	principal components analysis
SEM	Standard error of the mean
SD	standard deviation
SPL	sound pressure level
WL	Western Lowland
WM	Western Mountain

ACKNOWLEDGEMENTS

Firstly, I would like to thank my supervisor, Dr. Leslie Phillmore. As grateful as I am for her research guidance and feedback on the studies that make up my thesis, I am also extremely thankful for her encouragement and her great patience. Thank you for sticking with me and giving me the chance(s) needed to succeed.

I would also like to thank my committee, Drs. Dennis Phillips, Marty Leonard, and Kazue Semba. Their feedback as I prepared my thesis was invaluable. So too was the positivity and encouragement they brought to my committee meetings, which motivated me to keep working. I would also like to thank Andy Horn for his valuable feedback on my chapters and his interest in my research. Thank you also to Dr. Jennifer Stamp for acting as my comprehensive projects committee chair and for frequently providing me with valuable advice.

I am very grateful for the help I have received over the years from the many people that have worked in Dr. Phillmore’s lab. They include Ashley Lockyer, Jordan Fisk, Tareq Yousef, Dominique Shephard, Heather McGillivray, Stephanie Martin, Lynn Johnson, Sean Aitken, Jacquelyn Cormier, Andrew Veysey, Kristy Newell, and Laura Roblee. Thank you to Jeff MacLeod for his assistance with hermit thrush field work, and to Emily Sangster for her assistance with extraction of climatic data for Chapter 5.

I would also like to thank Dr. Daniel Mennill for his assistance with preparation of stimuli for the studies detailed in Chapters 2 and 3. I am also very grateful to the Dalhousie Psychology department’s technical and animal care staff, with special thanks to Christopher Wright for his work on the implementation and maintenance of the operant booths, as well as his assistance with other computer-related catastrophes.

I am immensely thankful to my wife, Emily, for her unwavering love and support, which picked me up many times when I wanted to give up. Words cannot sum up my gratitude. Likewise, I would not have been able to succeed without the support and encouragement of my parents. Thank you for instilling in me a love for science and learning, as well as the determination required to reach this goal.

CHAPTER 1: INTRODUCTION

For centuries, birdsong has transfixed not only artists, inspiring countless poets and musical composers, but also biologists and naturalists. The study of birdsong was, in its early days, characterized by naturalists making observations in the field, often discriminating song features by ear and visualizing songs using musical notation. The science of birdsong was revolutionized when Thorpe (1954) introduced the use of sonograms (graphs showing sound frequency over time), which allowed for precise visual depiction of vocalizations. Since then, birdsong researchers have explored not just the vocal behaviour of songbirds, but also the behaviour's underlying genetics and neurobiology (Catchpole and Slater, 2008). In many cases, because of the similarities between birdsong and human communication, discoveries from birdsong research have made valuable contributions to our understanding of human speech, language and hearing (Bolhuis and Everaert, 2013).

Birdsong is most frequently studied in songbirds, or oscines, which belong to the suborder Passeri (within the order Passeriformes); they can be distinguished from other Passeriformes, or suboscines, and non-Passeriformes on the basis of the structure of their vocal apparatus and differences in vocal development (Ball and Hulse, 1998). One reason for songbirds' usefulness as a model species is the occurrence of vocal learning, which happens in a very limited number of vertebrate taxa besides humans, including songbirds (plus parrots and hummingbirds) and some other vertebrates, such as bats and cetaceans (Fitch and Jarvis, 2013). Most songbirds learn their songs early in development by listening to and memorizing the singing of adult tutors around them and then, with time and practice, they develop crystallized forms of their species' songs (Ball and Hulse, 1998). Vocal learning in humans and song learning in birds have several similarities, including an early phase characterized by malleable and inconsistent vocalizations (babbling in humans, subsong in songbirds) and a sensitive period during early life when individuals are most amenable to learning (Bolhuis et al., 2010).

There are also extensive similarities between the circuitry of the songbird brain, which can be divided into distinct pathways associated with song learning, song production, and auditory perception, and human brain pathways associated with speech, language, and hearing (Jarvis et al., 2005; Bolhuis et al., 2010). This apparent case of convergent evolution between human and songbird communication systems, and their neurobiological bases, makes songbirds an excellent model in which to study communication. For example, the importance of the gene FoxP2 was first recognized in humans, in a family suffering from a developmental speech disorder. Much of our subsequent understanding about the role of FoxP2 in vocal learning in humans (and other vertebrates that exhibit vocal learning) has resulted from research carried out in songbirds (e.g., Heston and White, 2015).

Songbird and human auditory processing abilities are similar in some ways and different in others. For example, both songbirds and humans exhibit maximum frequency sensitivity in the range of 2-5 kHz, although humans can hear a larger overall range of frequencies (Woolley, 2013). Pitch processing abilities also differ: while songbirds are better than humans at absolute pitch processing (Weisman et al., 2010), they are comparatively poor at relative pitch processing (Page et al., 1989). In addition, songbirds exhibit superior temporal discrimination to humans (Lohr et al., 2006).

In general, songbirds provide an ideal model for exploring the interactions between brain and behaviour, especially with respect to song production and perception. Songbirds offer certain advantages over other model species (e.g., mice and rats) in that they can be studied in both field and lab, providing the opportunity not just to observe and explain behaviour in a controlled environment but also to link that to behaviour in natural environments. Songbird research has also been an important contributor to understanding the effects of human activity and environment change on animal behaviour, as demonstrated by studies of anthropogenic noise effects upon birdsong and reproduction (Slabbekoorn and Ripmeester, 2008).

1.1 BIRDSONG PRODUCTION AND PERCEPTION

The vocal repertoire of songbirds consists of two main types of vocalizations, songs and calls, which differ from each other in terms of use and function. For example, as indicated above, songs are typically learned during an early sensitive period, although there are cases where at least some song elements appear to be innate or invented (e.g., wood thrush (*Hylocichla mustelina*): Whitney and Miller, 1987). In contrast, calls are most often innate, although there are again exceptions, such as the *chick-a-dee* call of black-capped chickadees (*Poecile atricapillus*: Hughes et al., 1998).

Whereas calls are delivered by both males and females, song is usually, though not always, delivered by the male of the species (Catchpole and Slater, 2003). Lastly, calls tend to be used year round for a variety purposes, serving to aid in individual recognition, mediate interactions between parents and offspring, maintain contact between conspecifics, and alert others to predator risks. In contrast, song is used mainly during the breeding season and has two very specific functions (Ball and Hulse, 1998). First, territorial birds (most often males) defend their territories vocally, interacting with nearby males, sometimes as a prelude to physical altercation (Catchpole and Slater, 2008). Second, birds sing to attract mates, with those potential mates often assessing the singer's quality and making breeding choices based on song features (Catchpole and Slater, 2008).

One of the most noteworthy things about birdsong – and one of the primary challenges related to its study – is the wide variety across songbird species with respect to the structure of song, as well as how it is used. For example, in some species, individuals repeat a single version of its song, or song type, over and over (e.g., ovenbird (*Seiurus aurocapilla*)), whereas individuals of other species exhibit repertoires containing dozens (e.g., marsh wren (*Cistothorus palustris*)) to thousands (e.g., brown thrasher (*Toxostoma rufum*)) of distinct song types (Catchpole and Slater, 2003). In some cases, songs are added and/or replaced each season over a bird's lifetime (e.g., European starling (*Sturnus vulgaris*), Eens et al., 1992). Song structure itself differs greatly across songbird species, ranging from a few simple whistles to longer, more complex combinations of notes. Songbird species differ just as dramatically in other aspects of singing behaviour, including song syntax (how species with multiple song types switch between those types), how song structure varies across geographic space, and how song is used to interact with other members of the same species. The two species studied in this thesis, discussed below, differ in many of these respects, including song complexity and repertoire size.

As much as vocal production, song perception is a vital aspect of song's role in breeding and reproduction among songbirds. In order for a singing male to recognize a potential territorial intruder or for a female to assess a potential male mate based on its song, an individual must first recognize that song as coming from a member of its own species. As with song structure itself, the song features used for species recognition differ extensively across species. For example, depending on the species, conspecific recognition may depend on the presence of certain song elements, such as the red-winged blackbird's (*Agelaius phoeniceus*) song-ending trill (Beletsky et al., 1980), or on more particular structural characteristics, such as the frequency relationships within and between notes in the song of the black-capped chickadee, discussed below.

Individual recognition is another important aspect of song perception, mediating how individuals choose to interact with each other during the breeding season. For example, according to the "dear enemy hypothesis" (Temeles, 1994), territorial males distinguish between neighbours and strangers based on song, and they base their response (i.e., the degree of aggression) upon this discrimination. Individual recognition is also important for female songbirds, which must be able to recognize differences between individuals in order to assess male quality on the basis of song structure and performance (e.g., Ballentine et al., 2004) and make reproductive choices on that basis (e.g., Menill et al., 2002).

1.2 STUDY SPECIES

This thesis uses two North American songbird species to study factors affecting both vocal production and auditory perception of conspecific vocalizations: the black capped chickadee and the hermit thrush (*Catharus guttatus*). These two species are similar to each other in that they exhibit characteristic songbird breeding behaviour, with males establishing and defending territories while trying to attract females for mating. However, as detailed below, they differ considerably in singing behaviour. For example, black-capped chickadee males sing a structurally simple song that varies little, while male hermit thrush sing approximately a dozen varieties of a song that is structurally more complex. Considering the overall variability in the forms and uses of birdsong, there is great value in studying multiple species, and especially in studying songbirds that differ with respect to characteristics such as structure, repertoire size, and syntax.

The black-capped chickadee is an extremely common, widespread songbird species, found in deciduous and mixed woods but also areas disturbed by human activity, including suburban backyards (Foote et al., 2010a). Non-migratory, the black-capped chickadee has a large distribution that covers much of North America and spans from coast to coast. During the winter, they travel in flocks that are governed by a male dominance hierarchy, with dominance among males determined primarily on the basis of age (Schubert et al., 2008). In the spring, male chickadees establish territories that they maintain throughout the breeding season. Males and females establish monogamous pair bonds during the breeding season, although extra-pair copulations occur, usually as a result of a female mating with a male ranked higher than its first partner (Smith, 1988; Mennill et al., 2004). As a common songbird with a simple song (Figure 1.1a), the black-capped chickadee is a frequently used model species for research on varied topics in birdsong, including seasonal brain changes (Phillmore et al., 2006), structure and function of calls (e.g., Charrier et al., 2004), and the particulars of song's role in reproductive behaviour (e.g., Otter et al., 1998).

Chickadees have a single, simple song type (phonetically described as *fee bee*) that can vary in absolute frequency but features consistent relative pitch ratios between the beginning and end of the *fee* note and between the *fee* and *bee* notes (Weisman et al., 1990). Song structure is remarkably conserved across much of the species' range (Kroodsma et al., 1999), and the ability to accurately maintain these pitch relationships may be an indicator of male dominance and/or quality (Christie et al., 2004b). Research has demonstrated that male singing performance influences chickadee reproductive behaviour: females eavesdrop on male countersinging (i.e., listen to males that are vocally interacting through song) and make decisions related to reproductive behaviour based on the outcome of such contests (Mennill et al., 2002).

Studies in chickadees have demonstrated differences in neural activity in response to conspecific versus heterospecific vocalizations within brain areas associated with auditory perception, with these differences modulated by breeding status (Phillmore et al., 2011). While behavioural studies have shown that male and female chickadees respond more strongly to normal songs compared to songs altered with respect to their frequency ratios, little research attention has been paid to the brain activity that underlies these differences. Chapter 1 addresses this question through an experiment assessing both behavioural and neural responses of male and female chickadees to songs with species-typical pitch ratios and songs with those ratios altered. In Chapter 2, I use the same song stimuli within an operant discrimination paradigm, expanding upon the first study by exploring the abilities of male and female chickadees to discriminate between normal and altered songs.

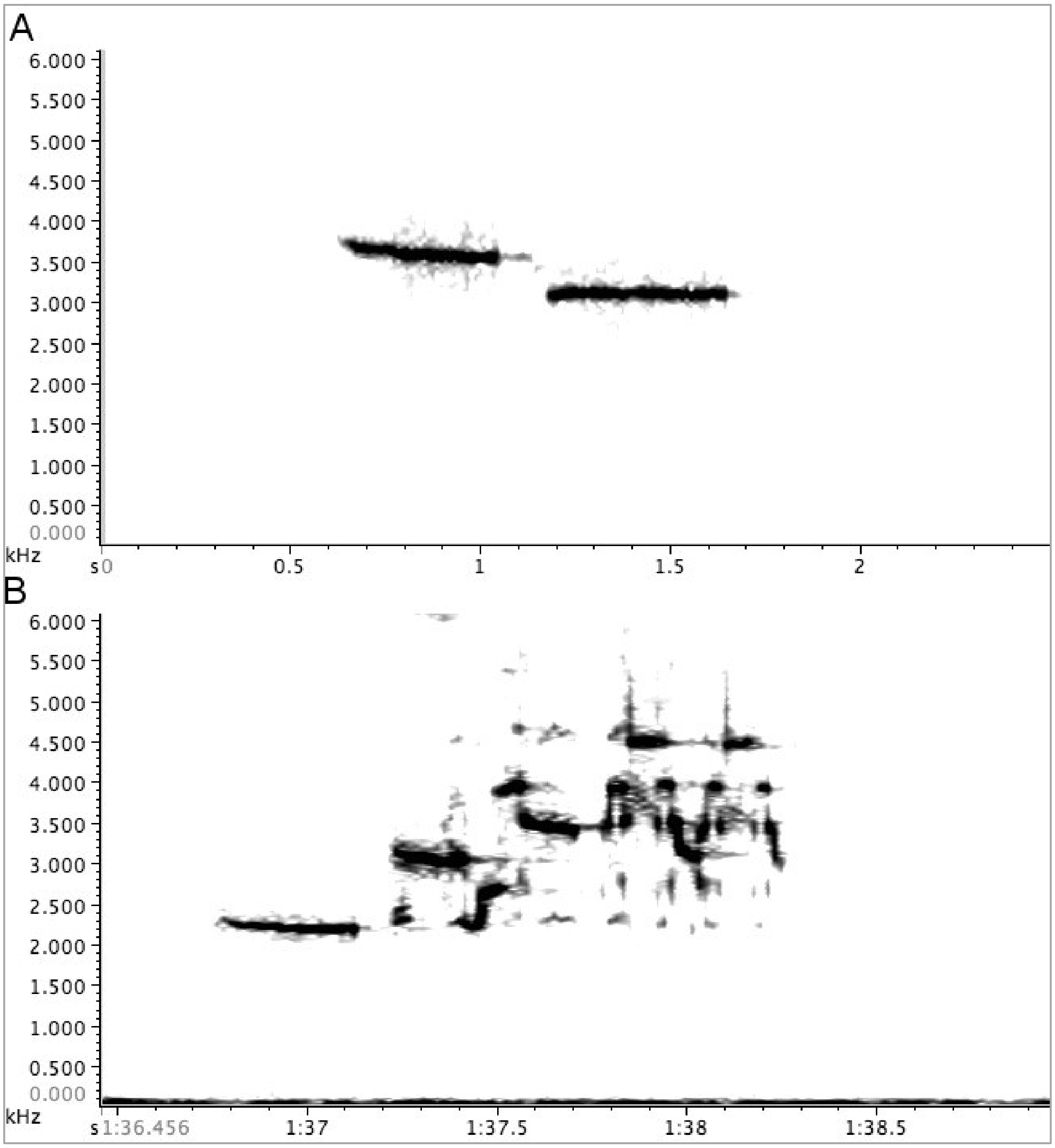

Figure 1.1 Example spectrograms showing representative songs of the black-capped chickadee (A) and hermit thrush (B)

The hermit thrush is a migratory, territorial songbird that breeds in coniferous and mixed woods across much of Canada, as well as the western and north-eastern United States, and winters in the southern United States and south into Central America (Dellinger et al., 2012). Migratory patterns vary: birds in the south-western United States are typically altitudinal migrants that go from low to high elevations within the same region for breeding, while other groups travel relatively long and circuitous routes that appear to relate to the species' evolutionary past (Dellinger et al., 2012; Alvarado et al., 2014). Unlike the black-capped chickadee, which is quite homogeneous across its distribution with respect to song, genetics, and morphology (Foote et al., 2010a), the hermit thrush varies greatly across its dozen or so subspecies with regard to vocal repertoire and morphology. Recent evidence also suggests a high degree of genetic divergence between eastern and western subspecies (Alvarado et al., 2014).

Hermit thrush singing behaviour differs substantially from that of black-capped chickadees. As is evident from the phonetic description (*oh, holy holy, ah, purity purity eeh, sweetly sweetly*), hermit thrush song structure is considerably more complex than the simple two-note song of the chickadee, with an introductory whistle followed by an intricate flourish of shorter notes (Figure 1.1b). Hermit thrush also differ with respect to repertoire size, with each individual employing 6-12 song types that differ considerably not just in absolute frequency but also in note organization (Rivers and Kroosma, 2000). Song structure is diverse not only within individual repertoires but also between individuals, as there is no evidence that neighbouring males share identical song types (Rivers and Kroodsma, 2000; Jones, 2005).

Given its moderate repertoire size, variation in structure, and separate song components (introductory and post-introductory portions), the hermit thrush is an ideal species in which to study topics such as species and individual recognition, the function of song repertoires, and the biological significance of distinctive song components. However, despite being a common songbird with a well-known song, little study has focused on hermit thrush singing behaviour. Chapter 3 aims to fill in such gaps by examining repertoire size, the characteristics of the song types making up repertoires, and the syntax with which birds use those song types.

Chapter 4 expands on such basic knowledge of singing behaviour, as well as limited information on geographic song variation (Rivers and Kroodsma, 2000), by conducting a thorough overview of geographic variation in hermit thrush song structure. The final chapter builds on the analysis of geographic song variation through a playback experiment comparing conspecific recognition via male territorial responses to local song and to song from a distant subspecies. These findings are discussed in the context of recent research showing genetic and morphological divergence between eastern and western lineages that is suggestive of a possible species split.

Thus, overall this thesis offers new insights into vocal production and perception using two species that differ extensively with respect to singing behaviour. In one, the black-capped chickadee, I examine specific aspects of perception within a well-studied song system. In contrast, this thesis's studies with hermit thrush establish essential information about song structure and song syntax, while also providing the first information about species recognition and the roles of the song's components in a rarely studied song system. In doing so, I lay the groundwork for more detailed studies of hermit thrush song, which will eventually provide vital insights into birdsong from a perspective that is distinctive from, yet complementary to, knowledge of birdsong in species such as the black-capped chickadee.

CHAPTER 2: BEHAVIOURAL AND NEURAL RESPONSES TO NORMAL AND ALTERED SONGS BY BLACK-CAPPED CHICKADEES (*POECILE ATRICAPILLUS*)

2.1 INTRODUCTION

Used primarily by male songbirds, birdsong plays a vitally important role in the breeding behaviour of both males and females (Catchpole and Slater, 2008). Among male songbirds, song serves as a key aspect of territorial defense against male conspecifics and is thought to play a role in aggressive interactions between rival males (Searcy and Beecher, 2009). In the case of females, male song quality is used as an indicator of the singer's quality and thus plays a role in mate selection. For example, according to the developmental stress hypothesis, song characteristics can indicate overall male quality by reflecting how well song was learned during early development, a time of great stress and resource demands (Nowicki et al., 1998). Research has shown that female songbirds show a stronger reproductive response to males with better developmental histories, which is reflected in their song attributes (Searcy et al., 2010).

As part of their large vocal repertoire, black-capped chickadees (*Poecile atricapillus*) produce a simple song consisting of a note that decreases in frequency over its length (*fee*) followed by a note of constant frequency (*bee*) (Ficken et al 1978, see Figure 2.1a). This song, phonetically named *fee bee*, is sung primarily by males (although females also rarely sing a *fee bee* song (Hahn et al., 2013b)) and is used in aggressive and sexual contexts as described above, including the dawn chorus. While varying in absolute frequency, the *fee bee*'s relative frequency structure adheres to two pitch ratio rules that are uniform among the vast majority of North American chickadee populations (Kroodsma et al., 1999). Specifically, the pitch differences between the start and end of the initial *fee* note (a downwards inflection referred to as a *glissando*) and between the end of the *fee* and the start of the *bee* (often referred to as the inter-note interval) are governed by highly consistent pitch ratios (1.056 and 1.134, respectively) (Weisman et al., 1990, see Figure 2.1a).

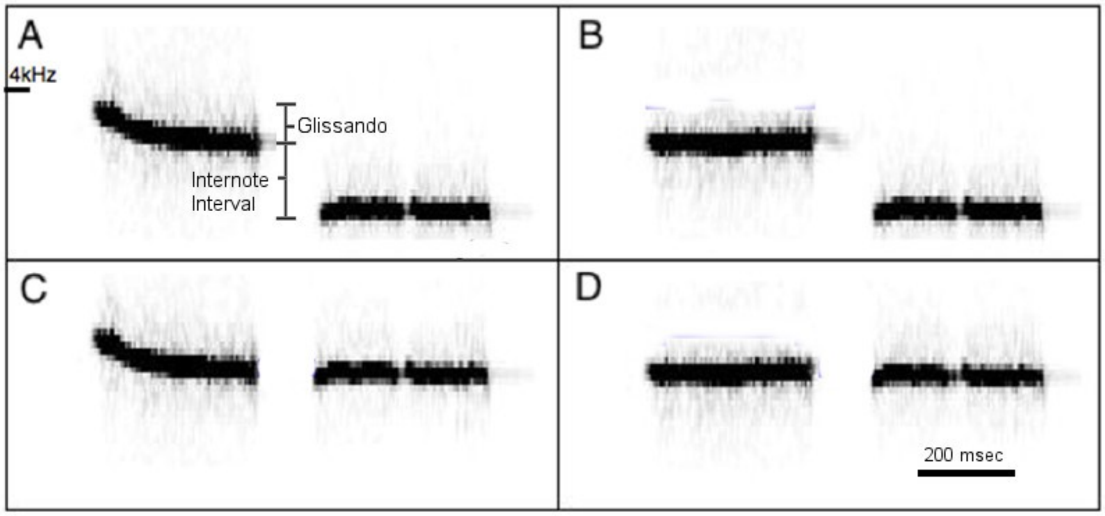

Figure 2.1 Spectrograms showing the stimuli used: Normal (with species-typical pitch ratios labeled) (A), Flat Fee (B), Flat Interval (C), and Flat Fee / Flat Interval (D).

Both male and female black-capped chickadees appear to be influenced by singing heard during dawn chorus. Chickadees can discriminate among individuals based on song characteristics (Christie et al., 2004a; Wilson and Mennill, 2010). During the dawn chorus, males interact vocally in large communication networks, listening to the singing of others and modifying their interactions with rival males in response (e.g., changing the degree to which they sing songs that match the frequency of conspecific songs) (Foote et al., 2010b; Baker et al., 2012; Toth et al., 2012). Female chickadees eavesdrop on these male vocal interactions during the dawn chorus, and information gathered from this eavesdropping influences reproductive behaviour (e.g., extrapair copulations) (Mennill et al., 2002). Dominant, or high-ranking, males have greater reproductive success (Otter et al., 1998), and this difference may relate to assessment of male quality via song structure: dominant males are better able to achieve the species-typical pitch ratio between the *fee* and *bee* notes (Christie et al., 2004b).

Previous behavioural studies have demonstrated the specific importance of the pitch ratio rules characterizing the *fee bee* song via playback experiments using songs with species-typical pitch ratios and songs in which those pitch ratios have been altered. In a field playback study comparing behavioural response to typical and altered song, male chickadees did not display as strong a territorial response to songs lacking the *glissando* change in the *fee* note, indicating that the *glissando* is important for male recognition of conspecific song (Shackleton et al., 1992). In contrast, male chickadees do not show different territorial responses to songs with the inter-note interval altered (Ratcliffe and Weisman, 1992; Shackleton et al., 1992). In a lab experiment, Ratcliffe and Otter (1996) found that females exhibited more sexual solicitation displays to normal songs than to songs with either the inter-note interval altered or the *glissando* inflection removed. Thus, in terms of behavioural response, it appears that females attend to both of the pitch ratio rules characterizing male song, whereas males attend only to the *glissando*. However, this does not appear to be due to a deficit in the ability to recognize the differences. Njegovan and Weisman (1997) used an operant discrimination paradigm to demonstrate that male chickadees are capable of discriminating among different inter-note pitch ratios. In that study, males were able to successfully discriminate between rewarding (S+) stimuli featuring an inter-note ratio of 1.12 (close to the species-typical ratio of 1.134) and non-rewarding (S-) stimuli with ratios of 1.00 and 1.26. I wondered, then, if the behavioural differences and perceptual performance described above would be reflected in changes in responsiveness within avian brain regions associated with the perception and recognition of auditory input.

Auditory information is processed in the songbird brain via an ascending pathway that is analogous to the brain circuitry governing human auditory perception, and which makes connections with the pathways that control vocal learning and production in songbirds (Pinaud and Terleph, 2008). Within the ascending auditory pathway, caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM) appear to play particularly important roles in classifying acoustic information. For example, electrophysiological studies have demonstrated that cells in these areas respond more to conspecific vocalizations than to heterospecific vocalizations (Chew et al., 1996) or other, non-avian sounds (Grace et al., 2003). Similarly, lesions to CMM interfere with zebra finches' (*Taeniopygia guttata*) ability to discriminate between conspecific and hetereospecific vocalizations (MacDougall-Shackleton et al., 1998).

Activity in NCM and CMM in response to acoustic stimuli is most commonly measured by quantifying the number of cells that express the immediate-early gene ZENK via immunohistochemistry (London and Clayton, 2010). This neural response, in black-capped chickadees and other songbirds, varies depending on a number of factors related to characteristics of both the vocalization and the listener. For example, ZENK expression to conspecific vocalizations is typically higher in male chickadees than in female chickadees (Phillmore et al., 2003; Avey et al., 2008). Sex of the singer also affects neural response, with greater ZENK expression to chickadee songs produced by males compared to those produced by females (Avey et al., 2008). Phillmore et al. (2011) demonstrated a seasonal difference in ZENK expression: ZENK immunoreactivity within the dorsal NCM of male chickadees was higher in response to conspecific compared with heterospecific vocalizations, but only when the chickadees were in breeding condition. In a recent experiment by Avey et al. (2014), ZENK expression in NCM and CMM did not differ in response to conspecific calls compared to heterospecific calls of similar acoustic characteristics, from zebra finches (*Taeniopygia guttata*), chestnut-backed chickadees (*Poecile rufescens*), and tufted titmice (*Baeolophus bicolor*), suggesting that differences in reactivity result primarily from differences in the structural characteristics of the vocalizations, regardless of whether or not they happen to be conspecific.

The objective of this study was to examine the behavioural and neural responses of black-capped chickadees to songs with typical pitch ratios compared to songs with altered pitch ratios. In particular, I wanted to see if immediate early gene (ZENK) activity in NCM and CMM corresponds with the behavioural differences observed in response to normal and altered songs. Further, in light of the sex differences suggested by behavioural work, I sought to determine whether males and females differed with respect to amount of ZENK expression in response to these songs. I hypothesized that ZENK expression to normal and altered stimuli would reflect previously observed behavioural differences; specifically, I expected ZENK expression in males to be higher in response to normal songs compared to songs with the *glissando* eliminated, and in females to be higher in response to normal songs compared to songs with either of the pitch ratios altered.

2.2 METHODS

2.2.1 Animals

Adult black-capped chickadees (n = 24 males, 23 females) were captured within the Halifax Regional Municipality between 25 November 2011 and 16 February 2012 (CWS Permit # ST2779). Individuals were attracted via playback of chickadee vocalizations (including calls and songs, recorded in Ontario at least a decade prior to this study) and captured using potter traps baited with sunflower seeds. Sex of the bird was assessed at time of capture based on mass, wing chord and tail length (Desrochers 1990), and confirmed by examination of gonads at perfusion. Age of the bird was determined by the extent and shape of white in the outer retrices: in non-adult birds the white coloration extends along one side of the retrices and terminates at the tip, while in adult birds the coloration extends around the top to the other side (Pyle, 1997). Non-adult (hatch-year) birds were released immediately; the age of adult birds was confirmed at perfusion by looking for skull pneumatization, which is exhibited only by adult birds. In order to minimize stress, birds were transported to individual cages at Dalhousie University's animal care facilities within 1 hour of capture. In addition, all birds were caught prior to noon in order to provide adequate habituation time in animal care prior to the end of the first day. All methods and procedures in this study were approved by Dalhousie University's University Committee on Laboratory Animals (Protocol # 08-020).

2.2.2 Housing and Feeding

After capture and transport, birds were placed in individual cages (91 cm wide x 41 cm high x 46 cm deep) made of galvanized steel mesh with a stainless steel pan for collecting waste. Each cage contained wooden perches, a swing and a water bath, as well as evergreen boughs, and birds had *ad libitum* access to drinking water, grit and food (a mix of husked sunflower seeds and Mazuri Small Passerine diet feed, with unhusked sunflower seeds on top). Birds were housed together in rooms containing 10-15 birds each, and the initial light cycle in each room was set to approximately reflect natural day length at time of capture. Room temperatures were maintained at approximately 18°C.

2.2.3 Photostimulation

All birds were photostimulated in order to bring them into breeding condition. To allow habituation to animal housing, photostimulation was initiated a minimum of 7 days after capture. Following habituation, lighting was altered to an extended “daylight” schedule (15 hours light: 9 hours dark) to reflect day length during the early spring and to bring the birds into breeding condition (Phillmore et al., 2005). Each bird was photostimulated for 5-6 weeks prior to the experiment.

2.2.4 Experimental Apparatus

The experimental apparatus, to which each bird was transferred after photostimulation, consisted of a standard budgerigar cage (47 cm wide x 30 cm high x 30 cm deep) containing drinking water, grit and food (same mix as above), as well as three wooden perches positioned at equal height and distributed equidistantly along the longest dimension of the cage. The cage was housed on a shelf within a sound-attenuating booth (Eckel Industries, Model #AB2000). The shelf also contained a speaker (Cambridge Audio Atom V.5; response range 5-20,000 Hz) aimed directly at one end of the cage and connected to an iPod nano (Apple) via an amplifier (Cambridge Audio Azur 340A.340A SE), both of which were located outside the booth. A video camera (Sony Model # DRC-HC28), attached to an adjustable steel rod suspended near the top of the booth and connected to an iMAC (equipped with iMovie for video/audio recording during playback) outside the booth, was pointed directly down on the cage in a “bird’s-eye” view. The speaker and the video camera were connected to their external components through a panel located on the interior wall of the booth. Prior to a bird being transferred to the booth, amplitude of the selected playback recording was adjusted to 70dB SPL (at the perch closest to the speaker) using a decimeter (Extech Instruments, model #407750). The booth was lit by four cool white 20-watt fluorescent light bulbs (Sylvana) located against the outer surface of a window in the booth’s wall that was cased in, so that light within the booth could be controlled. The light cycle in the booth matched the schedule for photostimulation (15 hours light: 9 hours dark) and the temperature averaged 18°C.

2.2.5 Stimulus Vocalizations

Stimulus vocalizations for playback consisted of *fee bee* songs of four different types: Normal (species-typical frequency ratios between the start and end of the *fee* note and between the end of the *fee* and start of the *bee*); Flat Fee (frequency ratio from start to end of *fee* note reduced to 1.0); Flat Interval (frequency ratio between end of *fee* and start of *bee* reduced to 1.0); and Flat Fee/Flat Interval (both frequency ratio alterations). The frequency ratio (1.0) selected for the alterations to frequency ratios lies outside the typical range of pitch ratios (Christie et al, 2004b). The unaltered recordings consisted of six *fee bee* songs taken from the same set of recordings originally made between February 1999 and May 2000 that was used in previous work (Phillmore et al. 2003; 2011). They ranged in absolute frequency (measured at beginning of the bee note) from 3100-3350 Hz, so as to reflect the normal range of *fee bee* song frequencies (Weisman et al., 1990); each stimulus began at a different frequency. Stimuli for the three other playback groups were prepared by altering the frequency ratios in the three ways described above of each of the original songs using the audio editing program Audition 2.0 (Adobe). This gave me a total of 24 song stimuli (6 for each playback group) so that every potential subject for a given sex would hear a unique playback stimulus.

2.2.6 Playback

Following photostimulation, each bird was transferred to a standard budgerigar cage inside one of four sound-attenuated booths (described above) during the morning of the day prior to the experiment. Although the booths were located in the same room, sounds from one could not be heard inside another when both doors were closed. The door of each booth was kept open during the day of transfer to minimize stress during habituation and then closed overnight. The next day, following a total habituation time of approximately 24 hours, each bird was videotaped for 90 minutes, consisting of 30 min each of pre-playback, playback and post-playback. Prior to the 90 minute period, the door of the booth was opened briefly and quietly in order to turn on the video camera (more than one booth was never open at the same time). Following the 30 min pre-playback period, the bird was presented with 30 min playback from one of the four playback groups described above, during which the stimulus song was repeated every 10 seconds. Assignment of birds to playback groups was random with the restriction that no two birds of either sex had the same stimulus. Playback was scheduled to take place after 0900 and before 1200 for all birds. Video recording continued during a 30 min post-playback period following the end of playback, and 60 min after the conclusion of playback the bird was removed from the booth and euthanized, in keeping with previous protocols for ZENK protein immunohistochemistry following playback (Phillmore et al 2003; 2011).

2.2.7 Perfusion

Thirty minutes after the end of the post-playback recording period, each bird was deeply anaesthetized using 0.15 cc of Euthanyl. Following this, each bird was transcardially perfused with 0.1 M phosphate-buffered saline (PBS) and then with 4% paraformaldehyde for 3-5 min each. The brain was removed and post-fixed in 4% paraformaldehyde for 24 hours, followed by cryoprotection in 30% sucrose solution (in PBS) for about 24 hours (until saturation was reached). Brains were flash frozen using dry ice and stored at -80°C until sectioning. At the end of each perfusion, sex was confirmed by examination of the gonads. For each male, the length and width of the left testis was measured to the nearest 0.1 mm using dial calipers; testis size differs dramatically between breeding and non-breeding males (Phillmore et al., 2005). For these data, I estimated the testis volume using the formula 4/3_a2b (where a = width/2 and b = length/2). In females, the ovaries were examined to confirm breeding condition, which can be determined on the basis of the presence and extent of hierarchical follicles on the surfaces of the ovaries (Phillmore et al., 2005).

2.2.8 ZENK Immunohistochemistry

Each brain was divided into separate hemispheres and sectioned sagittally at 30 um using a cryostat. Every third section from the midline was used in immunohistochemistry for ZENK; if being processed immediately, sections were collected into PBS. If not being processed right away, sections were collected into cryoprotect (30% sucrose and 30% ethylene glycol in buffer) and stored at -20°C. Immunohistochemistry was performed on the free-floating sections, beginning with washes in 0.1 M PBS, incubation in 0.5% hydrogen peroxide (15 min), and additional washes in 0.1 M PBS. Sections were incubated in 10% Normal Goat Serum (Vector) and then incubated in a commercially available primary antibody raised in rabbit (anti Egr-1, Santa Cruz Biotechnology, catalog # sc-189; 1:20,000 dilution in PBS containing 0.3% Triton-X) for approximately

20 hours. Sections were then washed in PBS containing 0.1% Triton-X and incubated in biotinylated goat anti rabbit IgG antibody (Vector; 1:200 dilution in PBS containing 0.3% Triton-X) for 1 hour. Sections were washed again in PBS containing 0.1% Triton-X and then incubated in avidin-biotin horseradish peroxidise (Vector ABC Vectastain Elite kit) for 1 hour. After washing again in 0.1 M PBS, labeling was made visible using SigmaFast DAB tablets (3',3-diaminobenzidine tetrachloride) (Sigma). Sections were mounted on gelatin-coated slides, dehydrated, and coverslipped using Permount.

2.2.9 Behavioural Analyses

During the 90 min recording period (before, during and after playback) for each bird, behavioural responses that are analogous to those used in playback field experiments were measured: proximity to the sound source (speaker), activity (time moving), and vocal responses. For vocal responses, the number and type of vocalizations (*fee bee* songs, *chick-a*-dee calls, *tseet* calls, gargle calls, and individual notes (e.g., *fee* notes)) made during each of the three recording periods were recorded.

Location within the cage was measured throughout the 90 min recording using the arena mode in EthoVision (Noldus). For the purpose of quantifying each bird's location, the arena (recorded from above) was divided into three equally-sized zones along the length of the cage with Zone 1 being the third of the cage closest to the speaker and Zone 3 being the third furthest from the speaker. Using EthoVision, the time spent in each of the zones during each of the three recording periods was calculated. Ethovision was also used to calculate the activity level of each bird during the 90 min recording, quantified as the total distance the bird flew (in cm).

2.2.10 ZENK Immunoreactivity Quantification and Analyses

The level of ZENK protein immunoreactivity (ZENK-ir) in three brain regions (the dorsal and ventral sections of the NCM, and the CMM) that exhibit neural activity associated with auditory discrimination (e.g., conspecific vs. heterospecific songs) (Chew et al., 2006) were measured. These regions were identified using borders and neuroanatomical landmarks used in previous papers (Phillmore et al. 2003, Avey et al. 2008). Specifically, the NCM was identified as lying caudal to Field L, while CMM was positioned rostral to the Field L and ventral to the lateral ventricle. For quantifying ZENK-ir in each brain, at least three sections per hemisphere that were located within 1080 μm of the midline (the first twelve sections collected) were selected. In order to ensure that orientation of Field L, and therefore identification of the regions of interest, was correct, only sections in which the NCM was still attached to the rest of the brain were selected; the same sections were used for CMM.

Images of each brain region were captured at 20x magnification using a Q-Imaging Fast 1394 digital camera mounted to an Olympus BX51 microscope. Analysis of the image was conducted using the software package Image-Pro Plus. On each image, an area of interest of size 2.71 mm^2 was drawn to demarcate the region being quantified and the detection threshold was adjusted manually so that only ZENK-positive cells were being counted. In order to avoid counting selected debris and/or overlapping cells, a size range restriction (9.07 μm^2 to 27.21 μm^2 [Avey et al., 2008]) was set. The experimenter doing the cell counts was blinded to the identity of the birds (i.e., to which experimental group they were in) until counting was complete. In the case of each region within each hemisphere, the counts for the three sections used were averaged.

2.2.11 Statistical Analysis

All statistical analyses were conducted in IBM SPSS Statistics 22 (Windows). Mixed analyses of variance (ANOVA) were used, with a Greenhouse-Geisser (GG) correction applied when the data failed Mauchly's Test of Sphericity.

2.3 RESULTS

2.3.1 Behaviour – Vocalizations

For each bird, I counted instances of each vocalization type separately, but found that the overwhelming majority of vocalizations (97.1% across all birds) were "tseets"; this is consistent with another playback study performed in our lab (Phillmore et al 2011). Although tseets were not the only vocalization produced, only seven out of 24 males produced a *fee bee* songs (three in the 'Normal' group, three in 'Flat Fee/Flat Interval', and one in 'Flat Interval'); three females sang *fee bee* songs (two in 'Normal' and one in

‘Flat Interval’). A total of 22 out of 47 birds produced *chick-a-dee* calls: 12 were males and 10 were females, and in each case they occurred in all four playback groups. Given the overall low numbers of those vocalization types and their dispersed occurrences, I pooled the counts of vocalizations together across types for each bird prior to analyses.

Vocal responses differed across playback group, with birds vocalizing more in response to ‘Normal’ and ‘Flat Fee’ stimuli compared to ‘Flat Interval’ and ‘Flat Fee-Flat Interval’ stimuli (Figure 2.2). I ran a sex x playback group x playback period mixed ANOVA; because the vocalization data failed Mauchly’s Test of Sphericity ($p < 0.05$), a Greenhouse-Geisser correction was applied. The mixed ANOVA revealed no main effect of sex ($F(1,46) < 1$) or playback group ($F(3,44) = 1.504$, $p = 0.228$). There was a significant main effect of playback period ($F(1.427,45) = 14.02$, $p < 0.001$), with post-hoc tests revealing that birds vocalized significantly more during playback than they did before or after. There was also a significant interaction between playback group and playback period (F(4.280,57.06 = 3.399, $p = 0.013$), which I explored further by doing separate one-way ANOVAs for each level of these two factors.

One-way ANOVAs for each playback group revealed a significant effect of playback period on the total number of vocalizations in the ‘Normal’ ($F(1.143,46) = 8.845$, $p = 0.009$) and ‘Flat Fee’ ($F(1.175,46) = 13.405$, $p = 0.002$) groups; in each case,

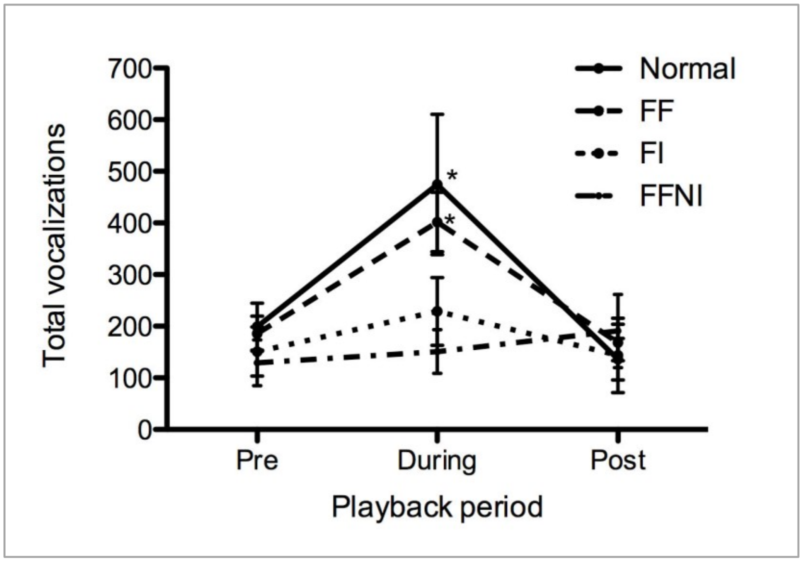

Figure 2.2 Mean total vocalizations (including all vocalization types) of birds during each playback period in response to Normal, Flat Fee (FF), Flat Interval (FI), and Flat Fee / Flat Interval (FFFI) stimuli. * indicates significant difference ($p < 0.05$) compared to other playback periods; error bars represent SEM.

post-hoc tests revealed that birds hearing these stimuli vocalized more during playback than either before or after. There was no effect of playback period for the ‘Flat Interval’ and ‘Flat Fee-Flat Interval’ groups. One-way ANOVAs for each playback period revealed no differences among playback groups in the periods before or after playback, but a significant effect of playback group during playback (F(3,44) = 3.861, p = 0.015). Post-hoc tests revealed that, during playback, birds that heard ‘Normal’ stimuli vocalized more than birds that heard ‘Flat Interval’ or ‘Flat Fee/Flat Interval’ stimuli, and birds that heard ‘Flat Fee stimuli’ vocalized more than birds that heard ‘Flat Fee/Flat Interval’ stimuli.

2.3.2 Behaviour – Proximity and Activity

Birds spent more time overall in close proximity to the speaker (Figure 2.3), although there was no difference in their location with respect to playback group or playback period. I conducted a sex x playback group x zone x playback period mixed ANOVA, applying a Greenhouse-Geisser correction because the data failed Mauchly’s Test of Sphericity (p < 0.05). There were no main effects of sex (F(1,47) = 2.890, p = 0.097), playback group (F(3,45) = 0.080, p = 0.970), or playback period (F(1.271,46) = 1.305, p = 0.269). However, there was a significant main effect of zone (F(1.692,46) = 13.887, p < 0.001); post-hoc tests revealed that birds spent more time in Zone 1 (nearest to the speaker) and Zone 2 than in Zone 3, but there was no difference between time spent in zones 2 and 3. There was also a significant interaction between sex and zone (F(1.692,65.99) = 3.769, p = 0.035), which I explored further by running separate one-way ANOVAs for each sex. In each case, there was a significant difference in the time spent in the three zones (males: F(1.45,31.98) = 9.995, p = 0.001); females: F(1.90,32.34) = 8.227, p = 0.002). Post-hoc tests revealed that males spent more time in Zone 1 and Zone 2 than in Zone 3, while females spent more time in Zone 1 than in Zone 2 and Zone 3 (Figure 2.3).

There were no significant differences related to activity, measured as the total distance flown (in cm) by each bird. I conducted a sex x playback group x playback period mixed ANOVA but there were no significant main effects of sex (F(1,39) = 1.27,

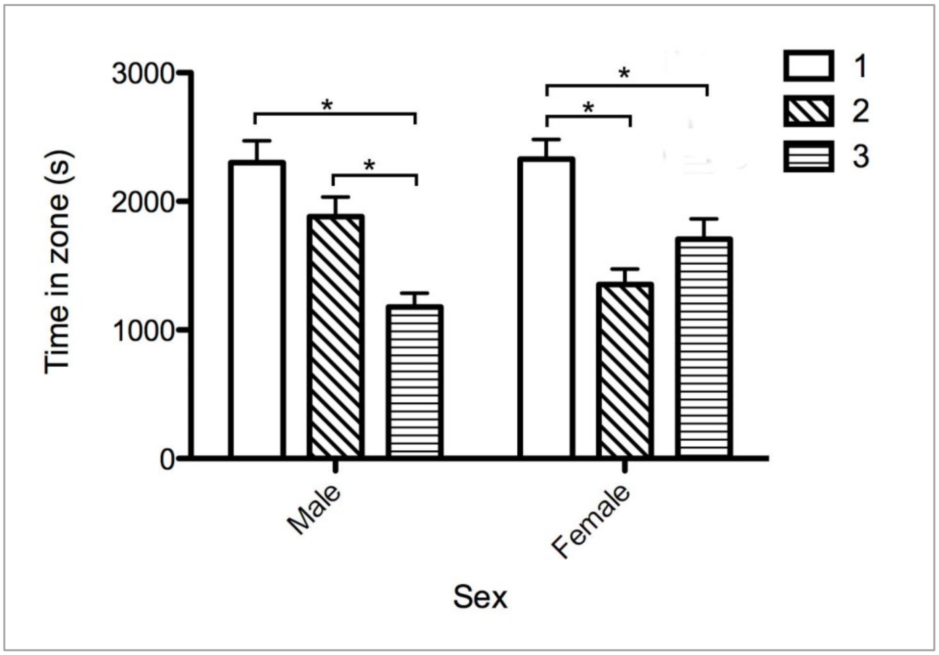

Figure 2.3 Mean time spent in Zones 1, 2, and 3 (ordered in increasing distance from speaker) by male and female chickadees, collapsed over playback group and playback period. * indicates significant difference ($p < 0.05$); error bars represent SEM.

p = 0.27), playback group (F(3,39) = 2.51, p = 0.07) or playback period (F<1), nor were there any significant interactions.

2.3.3 Physiology

The average testes volume for all male birds was 67.40 mm^3 (SD = 25.69), which is comparable to the testes sizes of breeding condition chickadees reported by other studies in our lab (e.g., Phillmore et al. 2006, 2011). A one-way ANOVA indicated that there was no difference in testes volume across the four playback groups (F(3,22) = 0.894, p = 0.460). All of the female birds had stage 2 hierarchical ovaries, indicating that they were in breeding condition.

2.3.4 Brain

Figure 2.4 shows an example of a section from which cell counts were taken in NCMd. The size of the area in which cells were counted with each region was kept consistent during the counting process. As a result, statistical analyses of ZENK expression were conducted using raw cell counts. Overall, the amount of labeling did not differ with respect to playback group within any of the three regions (Figure 2.5). We ran A sex x playback group x brain region (NCMd, NCMv, and CMM) x hemisphere mixed ANOVA revealed a main effect of sex (F(1,47) = 4.11, p = 0.049): females had more overall ZENK-ir than males. There was also a main effect of brain region (F(2,46) = 270.91, p < 0.001); post-hoc comparisons revealed that CMM exhibited significantly more ZENK-ir than both NCMd and NCMv, and that NCMd showed greater ZENK-ir than NCMv. There was no main effect of playback group (F(3,45) = 0.335, p = 0.800) or hemisphere (F(1,48) = 0.260, p = 0.613), nor were there any significant interactions.

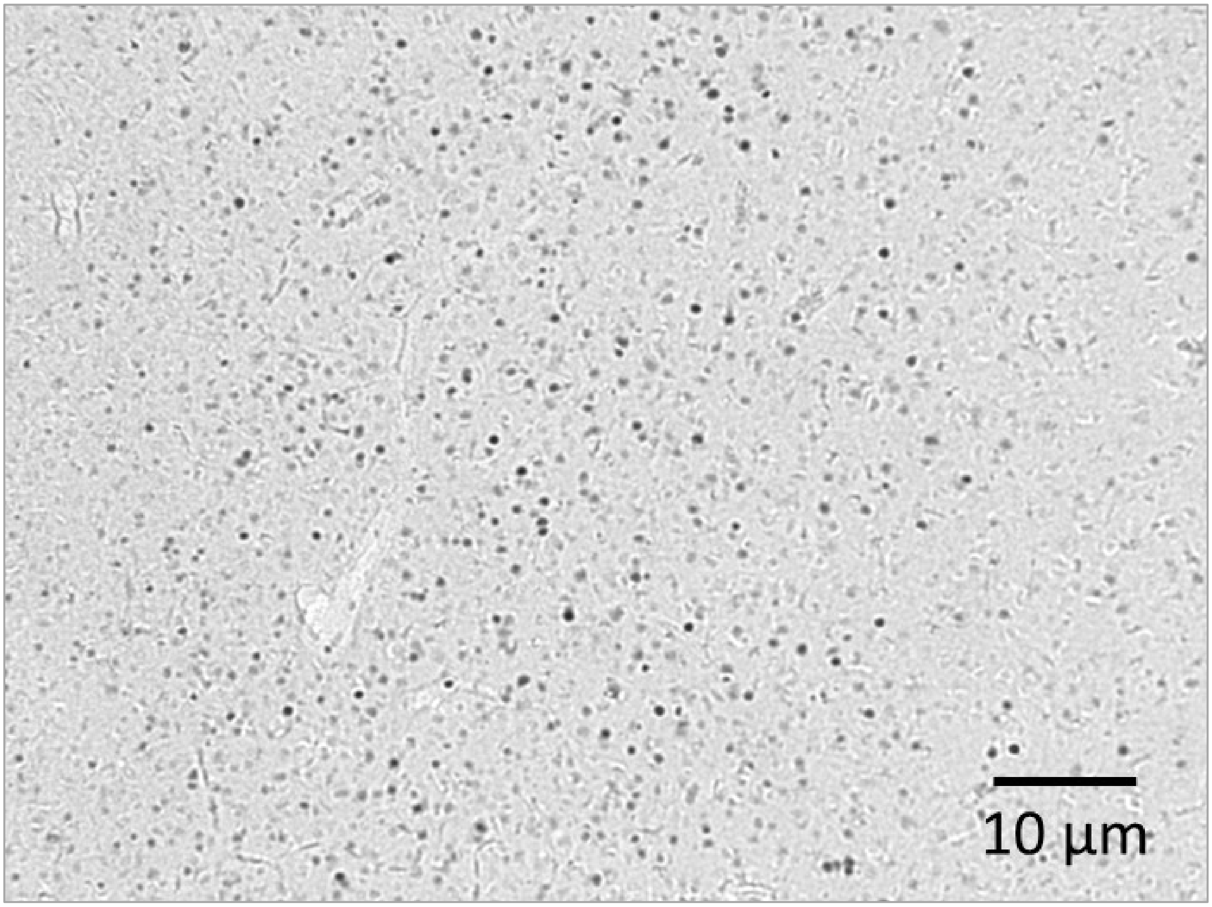

Figure 2.4 Representative example of ZENK labeling within the NCMd (20x objective lens).

Separate two-way (sex x playback group) ANOVAs were then conducted for each of the three brain regions examined, and because in the previous analysis there was no difference in cell counts between hemispheres, data from the left and right hemispheres were pooled for these analyses. In NCMd, there was no main effect of playback group

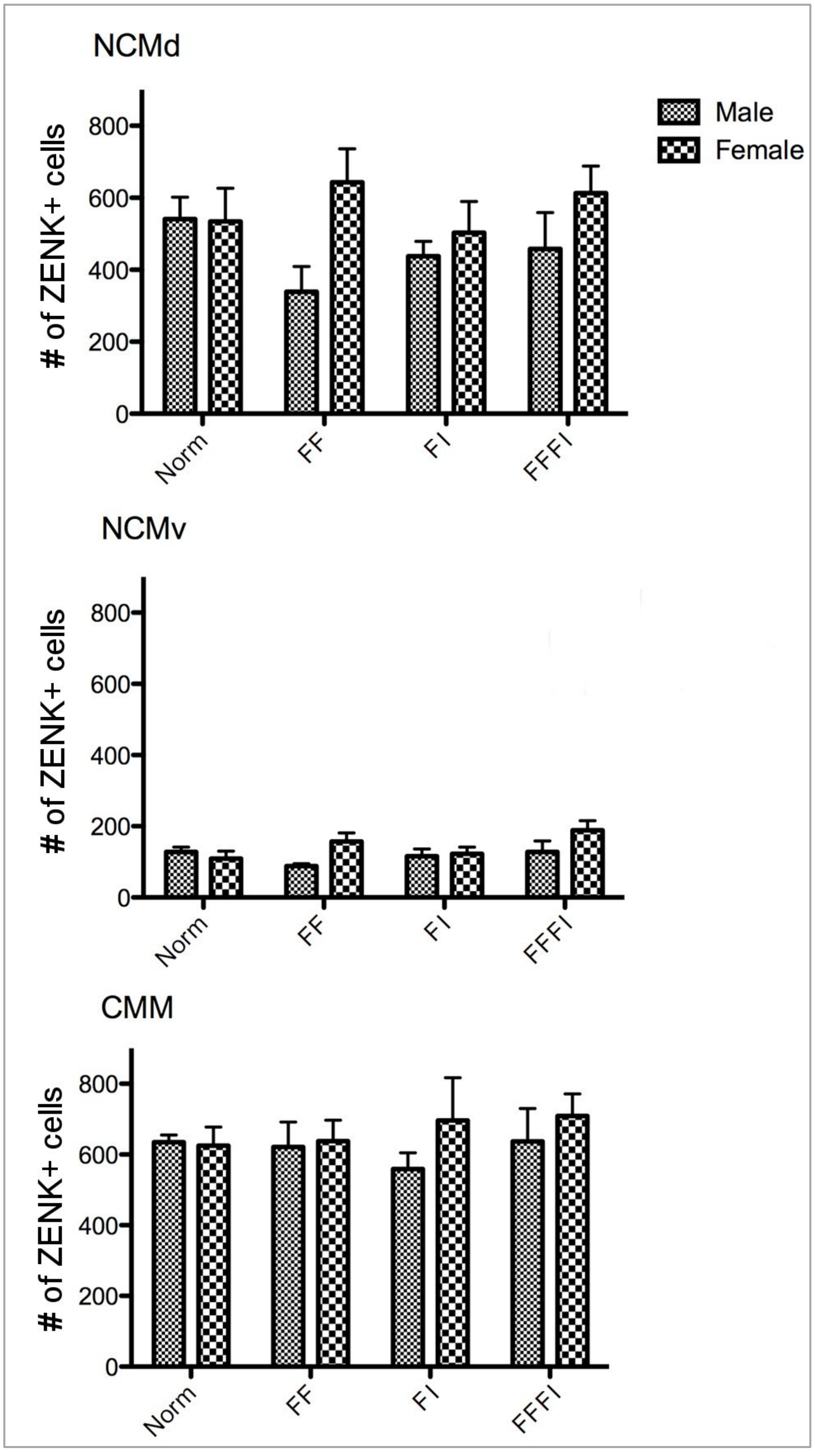

Figure 2.5 Mean number of ZENK-positive cells in male and female NCMd, NCMv, and CMM in response to four different types of auditory stimuli: Normal (Norm) (n = 7 males, 5 females), Flat Fee (FF) (n = 6 males, 6 females), Flat Interval (FI) (n = 6 males, 6 females), and Flat Fee / Flat Interval (FFFI) (n = 7 males, 6 females). Error bars represent SEM.

but there was a main effect of sex (F(1,47) = 5.190, p = 0.028); females had higher ZENK expression than males (Figure 2.5). For both NCMv and CMM, two-way ANOVAs revealed no main effects of sex (NCMv: F(1,47) = 3.665, p = 0.063); CMM: F(1, 47) = 1.231, p = 0.274)) or playback group (NCMv: F(3, 45) = 1.605, p = 0.203); CMM: (F(3,45) = 0.087, p = 0.967)) (Figure 2.5). Thus, the overall main effect of sex in the previous analysis was driven mainly by higher ZENK-ir in NCMd of females compared to males.

2.4 DISCUSSION

In this study, I sought to identify neural correlates for previously-reported behavioural differences in how male and female black-capped chickadees respond to songs with species-typical pitch ratio rules compared to those with those pitch ratios altered. Behaviourally, both males and females vocalized more in response to song with the inter-note interval intact (normal and flat fee) than to song with the interval removed (flat interval, flat fee/flat interval). However, general activity levels (distance flown), proximity to the speaker, and ZENK expression were not affected by altered playback. Males and females generally behaved the same way; there were no sex differences in amount of vocalizing, proximity to speaker, or activity. However, and perhaps most interestingly, females had more ZENK expression than males.

2.4.1 Behavioural Response to Playback

Previous field playback studies found that males exhibited a weaker territorial response to songs with the fee note's glissando removed (Ratcliffe and Weisman, 1992; Shackleton et al 1992), while a laboratory playback study found that females exhibit a weaker sexual response to songs with either the glissando or the inter-note interval removed. This was taken to be evidence that birds attend to alterations in the *fee bee* song's pitch ratio rules. More specifically, it suggested a sex difference in what parts of the *fee bee* song males and female chickadees attend to, with females attending to both pitch ratios and males only attending to the glissando.

While there were no differences with regard to activity levels or proximity the sound source in my study, birds vocalized more during playback than they did either before or after, indicating a significant behavioural response to the playback stimuli. For both male and females, this increase in vocalizations was observed only in response to normal songs and those with the glissando removed; birds did not vocalize more in response to songs with the inter-note interval removed (or to songs with both alterations). Thus, based on vocal responses to the stimuli, the inter-note interval was the important feature of *fee bee* song for both male and female chickadees in this study.

This finding is contrary to previous field work indicating that the glissando – and not the inter-note interval – is important to male chickadees. In one of these, a field playback study, Shackleton et al. (1992) found that territorial males responded more aggressively to playback of songs with normal pitch ratios than to songs with the *fee* note's glissando flattened, whereas increasing the inter-note interval made no difference. Similarly, Ratcliffe and Weisman (1992) found no change in territorial response by male chickadees when the inter-note interval was either eliminated (ratio changed to 1.00, as in the current study) or increased.

One explanation for the differences in male responsiveness between those studies and the current one is methodological: the previous findings resulted from field work conducted in the birds' territories, whereas the current study took place in a laboratory under more artificial conditions. Indeed, there are numerous examples, both in songbirds and other animals, of differences in behaviour and physiology between laboratory and field studies (Calisi and Bentley, 2009). Differences between lab and field studies have been demonstrated in black-capped chickadees, where captivity appears to influence whether or not certain song-related brain regions change volume depending on breeding condition (MacDougall-Shackleton et al.,2003; Phillmore et al., 2006; Smulders et al., 2006). Such effects of captivity have also been demonstrated in other songbirds, such as dark–eyed juncos (Smulders et al., 2000). Research in songbirds suggests that captivity can have profound influences on breeding behaviour, possibly via altered stress responses (e.g., starlings; Dickens and Bentley, 2014). In starlings, for example, fluorescent lighting affects stress levels and, following from that, behaviour (Evans et al., 2006; Evans et al., 2012).

Research in song sparrows demonstrates that stress during development can influence song selectivity during adulthood, with corresponding differences in neural responses (Schmidt et al., 2014). While such brain development-related effects would not apply to the adult chickadees used in this study, captivity and its associated stress may have nonetheless influenced their responsiveness. It is possible that the way in which the chickadees in this study responded to the normal and altered songs may have been influenced by the long period of captivity associated with photostimulation (5-6 weeks) and/or the small, isolated space of the sound-attenuated booth, within which they spent only one day of habituation prior to the experiment. Compared to the birds in previous field studies (Ratcliffe and Weisman, 1992; Shackleton et al, 1992), the captive males in this study, physiologically in breeding condition but in an entirely different (and unfamiliar) environment, may have attended to and assessed the song stimuli differently than they would have in a familiar territorial context. In particular, it is possible that the males in this study did not associate the *fee bee* songs as potential threats as they would have in the more natural context of their territories, and thus did not show, or could not show, typical responses. Field studies assessing the territorial responses of males to normal and altered *fee bee* songs in this population may help to clarify the reasons for the surprising behavioural responses seen here.

The decreased vocal response by female chickadees in this study to songs without the inter-note interval partly matches previous research examining female responses to altered songs (Ratcliffe and Otter, 1996). In their lab study, they measured the copulation displays of female chickadees in response to normal songs and to songs with alterations to each of the pitch ratios: they increased the typical inter-note interval ratio to 1.26 or, as in the current study, eliminated the glissando (reducing the ratio to 1.00). They found that, overall, females displayed more to the normal songs than to either of the altered forms, suggesting that female chickadees attend to both of the pitch ratio rules. Thus, our study agrees with the previous study in terms of female attendance to the inter-note interval, but did not find that females also attend to the glissando. Our study was fairly similar in methodology to Ratcliffe and Otter (1996), in that it involved catching chickadees from the wild, photostimulating them over several weeks, and testing them in sound-attenuated booths. However, it differed in some respects. In addition to differences in the timing and duration of playback, the behavioural measure was the number and duration of copulation displays by the females, something I did not record (or observe) in this study. It is possible that the different findings in our study related to such methodological differences. For example, Ratcliffe and Otter (1996) gave their female subjects estradiol implants. Another difference was that they presented each female with a frozen male chickadee as a model prior to presentation of the stimuli. It may be that, without such visual stimulation, the females in this study did not regard the stimuli as an indicator of a potential mate's quality, and therefore did not assess it as they would in the field.

Nonetheless, although Ratcliffe and Otter's (1996) study is the main source of information regarding which aspects of the *fee bee* song females attend to, the response differences observed were small and depended in part on how the stimuli were presented (Ratcliffe and Otter, 1996). The study's design involved playing each female chickadee a pair of songs (one normal and the other with one of the pitch ratios altered) on one day and then playing the same pair of stimuli in the opposite order on the next day. Their results were complicated by order effects. For example, birds exhibited longer displays to normal songs than to songs with an altered inter-note interval only when the normal song was the first stimulus presented; comparing normal songs to those with the *glissando* altered, females showed more displays to normal song only when the altered song was the first one presented. Given this, further studies – both in the field and the laboratory – are needed to further clarify which aspects of the *fee bee* song females assess for both species recognition and mate choice, especially in light of recent field studies suggesting that the inter-note interval in particular may play an important role in mating behaviour. Christie et al. (2004b) found that dominant, and therefore more preferred, males are better able to retain the species-typical inter-note interval, suggesting that it may be an indicator of male status used by females in mate selection. Research has also shown that the inter-note interval is relatively impervious to degradation due to broadcast through the forest, making it a good candidate for an accurate indicator of adherence to species-typical pitch ratios and, therefore, male quality (Christie et al., 2004b).

Another factor that may contribute to response differences between this study and previous ones is geographical location. In black-capped chickadees, structure of the *fee bee* song appears to be highly consistent throughout its range (Kroodsma, et al., 1999). However, there is some evidence of geographical differences with regard to how *fee bee* song is used to convey information. For example, Christie et al. (2004b) found consistency in the inter-note interval to be an indicator of dominance among chickadees in Ontario. In contrast, Hoeschele et al. (2010), studying a BC population, found that consistency in the relative amplitude of the *fee* and *bee* notes indicates dominance, with females responding more strongly to these 'dominant' songs. Follow-up work confirmed that relative amplitude aligned with dominance status in British Columbia, but not in Ontario (Hahn et al., 2013a). Thus, although chickadee song is geographically consistent in most respects, it is possible that the song features used for assessment of conspecifics vary with location. It may be, then, that the chickadees in this study differ from those in other studies with respect to how they attend to different structural aspects of the *fee bee* song. Christie et al. (2004b) demonstrated in an eastern Ontario population that, while the inter-note ratio remained relatively unaffected by broadcast through the forest, the glissando ratio changed significantly over increasing broadcast distances as a consequence of degradation. Differences in forest composition between Nova Scotia and eastern Ontario may lead to different degradation effects on *fee bee* song, in turn influencing which components chickadees rely on for recognition and dominance information.

There were no differences between playback groups with respect to proximity to the speaker or activity levels. The lack of behavioural differences, especially with respect to proximity, probably relates mainly to the relatively small size of the cage. A previous study in our lab using the same setup also found no differences related to proximity (Phillmore et al., 2011) and, although I increased the cage width for this study (from 40 cm to 47 cm), the cage was still relatively small. Given the dramatic difference between this arena and the area over which a bird might respond in the wild, it is not surprising that proximity does not provide an indication of response strength. Indeed, this stark difference likely impacts all of the behavioural measures.

2.4.2 Neural Response to Normal and Altered Song

The main objective of examining ZENK expression in the auditory regions CMM and NCM was to see if the previously described behavioural differences in response to playback of normal and altered song are reflected in neural responses within these auditory regions. Contrary to expectations, there was no overall effect of playback type on neural activity within these regions, suggesting that CMM and NCM do not respond differentially to *fee bee* songs with and without the species-typical pitch ratios.

There are several potential explanations for the lack of differences in neural response detected here. One possibility is that there are in fact differences in neural activity within CMM and NCM in response to these stimuli, but they cannot be detected using ZENK-ir. Avey et al. (2014) suggest with respect to a similar lack of differences in neural response to conspecific versus heterospecific calls in chickadees, there may be particular groups of neurons within these regions that are selective for the song features (pitch ratios) tested here. Such expression differences within regions would not be detected by the quantification used here, which measured the total ZENK expression within each region. Similarly, there may be subtle changes in the activity of neurons within NCM and CMM that cannot be detected via immediate early gene expression. Indeed, studies have demonstrated changes in electrophysiological activity in response to different aspects of conspecific vocalizations. For example, in European starlings, neurons in CMM fire differentially in response to different conspecific motifs (Meliza et al., 2010). Likewise, neurons in NCM of canaries and zebra finches are arranged tonotopically, responding differentially based on frequency (Terleph et al., 2008). Thus, it may be that brain activity related to discrimination of normal and altered *fee bee* songs is happening in these regions, but was not detected using this study's methods.

It is also possible that the relatively small changes in song structure distinguishing the normal and altered song stimuli in this study fall outside the discriminatory range of NCM and CMM, with such discriminations taking place at higher regions with the auditory system. Indeed, while abundant research has demonstrated the important roles of these regions in processing and extracting information from sounds, it has mostly been shown at the level of conspecific versus heterospecific vocalizations. This is the case in black-capped chickadees, where ZENK expression in the dorsal NCM of breeding birds is higher for conspecific vocalizations than for heterospecific vocalizations (Phillmore et al., 2011). There is some evidence of differential activity in CMM in response to different types of conspecific songs: Phillmore et al. (2003) found more CMM ZENK expression to songs than to calls. However, other studies found either no difference (Phillmore et al., 2011) or the opposite difference (Avey et al., 2008).

Studies in other songbird species also demonstrate a role for CMM and NCM in distinguishing between conspecific and heterospecific vocalizations. For example, in song sparrows, ZENK expression in both regions is higher in response to conspecific songs than it is in response to heterospecific songs (Schmidt et al., 2013). Likewise, CMM lesion in female zebra finches interferes with discrimination between conspecific and heterospecific vocalizations, highlighting its important role in species recognition (MacDougall-Shackleton et al., 1998). Electrophysiological work in zebra finches has shown that NCM neurons fire more to conspecific than to heterospecific vocalizations (Chew et al., 1996).

Recent work by Avey et al. (2014) further supports the idea that NCM and CMM may not be selective for fine structural features. They exposed black-capped chickadees to conspecific *dee* call notes as well as heterospecific calls of similar structure, from zebra finches (*Taeniopygia guttata*), chestnut-backed chickadees (*Poecile rufescens*), and tufted titmice (*Baeolophus bicolor*), and found no differences in ZENK response within NCM and CMM. These findings suggest that NCM and CMM are not so much selective for heterospecific versus conspecific vocalizations as they are selective for specific acoustic attributes of vocalizations. This selectivity would include the differences between the heterospecific and conspecific vocalizations typically used in such studies, as well as the differences between types of conspecific vocalization (e.g., chickadee songs versus calls), but not the more subtle differences distinguishing this study's stimuli. Research in other songbirds also supports a role for the NCM and CMM doing more fine-tuned forms of discrimination: ZENK expression correlates with song complexity in female budgerigars (Eda-Fujiwara et al., 2003) and with song length in female starlings (Gentner et al., 2000).

Thus it may be that song perception on the level of variation within conspecific stimuli involves other areas within the avian auditory pathway. For example, HVC, centrally important to song learning and production, may also contribute to fine discrimination among conspecific songs. In female songbirds, HVC lesions interfere with female assessment of male song quality in canaries (Brenowitz, 1991; Del Negro et al., 1998), and HVC neurons alter their firing patterns in response to so-called sexy syllables performed by male canaries (Del Negro et al., 2000). While this particular discrimination is female-specific (Del Negro et al., 2000), HVC may be otherwise involved in discrimination among conspecific songs.

2.4.3 Regional Differences

In this study, ZENK expression was highest in CMM, somewhat lower in NCMd, and lowest in NCMv. The relatively low reactivity in NCMv matches previous studies; however, the relatively high level of reactivity in CMM compared to NCMd differs with other research. For example, in chickadees, more ZENK expression has been seen in CMM than NCMd of chickadees in response to conspecific song (Phillmore et al., 2003; Phillmore et al., 2011), whereas this pattern of more reactivity in the chickadee CMM has been more typically observed in response to conspecific calls.

There is some previous evidence of higher reactivity in CMM in response to song in other species. For example, ZENK responsivity to conspecific songs was higher in the CMM of female European starlings compared to NCMd (Gentner et al., 2000; Sockman et al., 2005). In the current study, the high levels of ZENK expression in CMM may relate to the fact that all of the stimuli were conspecific vocalizations with small structural differences that did not appear to be distinguished at the level of NCM and CMM. Especially considering CMM’s apparent role in discrimination between conspecific and heterospecific vocalizations (MacDougall-Shackleton et al., 1998), it may be that the highly similar conspecific stimuli used in this study activated the CMM to a higher degree across all playback groups compared to other studies with stimuli that are discriminated at the level of CMM and NCM.

2.4.4. Sex Differences

In our study, there was a small but statistically significant sex difference in neural response: females exhibited more ZENK expression than males. This result contrasts with the findings of previous studies, in chickadees and other songbirds, in which ZENK expression in CMM and NCM has typically been higher in males than in females (e.g., Phillmore et al 2003; Avey, 2008). One difference between this study and previous ones that may contribute to these different findings relates to the stimuli used. In most studies, the stimuli used in most such studies induce differential neural responses; for example, heterospecific and conspecific vocalizations typically induce different levels of ZENK expression (Phillmore et al., 2011). Here, all of the relatively similar stimuli had the same robust effect on ZENK expression in NCM and CMM, possibly obscuring the typical sex difference in neural reactivity.

Another explanation for this unexpected sex difference may relate to how birds of each sex attend to and evaluate *fee bee* songs in a lab environment. As discussed above, both neural and behavioral responses often differ in lab experiments compared to what would be observed in a natural setting. In terms of attending and responding to *fee bee* song, it may be that this difference between field and lab is particularly pronounced for male chickadees because of the role it plays in territorial defense among males. By definition, the aggressive context in which males attend to the songs of other males often involves close physical (and visual) interaction among males, whether between adjacent territories or upon intrusion of one male into the territory of another. In contrast, such interaction is not as integral part of female assessment of male song. Indeed, female chickadees are more passive in that they listen to and assess the songs of several males at once, eavesdropping on singing contests between rivals (Mennill et al., 2002). The absence of a physical/visual component (i.e., a male chickadee) of playback may have decreased the salience of the auditory stimuli for the males in particular, thus contributing to comparatively lower levels of neural activity.

The potential effect of a laboratory environment upon neural responses is not unique to this study and therefore cannot fully explain the surprising sex difference observed here. However, the increased Zenk expression in female chickadees may result from an interaction between the unique set of stimuli – inducing a uniformly high neural response – and the effect of the lab environment on the salience of the stimuli to males, who are used to responding to song in aggressive circumstances characterized by close physical and visual interaction with conspecifics.

This study sought to determine the neural correlates for previous behavioural studies demonstrating that male and female black-capped chickadees respond differently to *fee bee* songs with and without particular species-typical pitch ratios. Behavioural responses to the normal and altered songs in this study partly matched the findings of those previous studies, but differed in some respects. Further research is required to understand the importance of these pitch ratio rules to both males defending territories and females selecting mates, especially considering the very limited amount of data upon which existing beliefs are based. Evidence of possible geographic differences in the relative importance of particular aspects of song structure underlines the need for more comprehensive study of how males and females assess *fee bee* songs.

The most notable result of this study is the overall lack of differences in neural responses to normal versus altered songs. Especially in light of the behavioural differences observed, this lack of a differential response suggests that, while auditory regions NCM and CMM are capable of discriminating the differences necessary for species recognition, more subtle discrimination (i.e., among conspecific songs with varying pitch ratios) occurs elsewhere in the avian auditory pathway. Thus, future studies in chickadees and other songbirds should look for differential neural responses to such stimuli within other nuclei, such as HVC.

Overall, researchers should endeavour to design studies that are capable of examining behavioural and neural responses within the same experiments, and in contexts that mirror the actual environment of the birds to the greatest degree possible. For example, the use of outdoor aviaries may allow for more robust testing of behavioural responses, allowing more space for males to engage in a territorially aggressive manner and opening up the possibility of carefully designed mate choice tests with females. Such studies have the potential to reveal more clearly the aspects of conspecific song upon which behavioural responses are based, while also allowing the neural responses underlying such behaviour to be examined.

CHAPTER 3: OPERANT DISCRIMINATION OF NORMAL AND ALTERED SONGS BY BLACK-CAPPED CHICKADEES (*POECILE ATRICAPILLUS*)

3.1 INTRODUCTION

Song plays a centrally important role within the breeding biology of passerine songbirds. Males use their songs for territorial defense during the breeding season, mediating aggressive interactions with potential intruders via singing behaviours such as overlapping and song type matching (Searcy and Beecher, 2009). Females assess potential mates based on their songs, using aspects such as repertoire size, adherence to species-typical song structures, and the ability to sing physically demanding songs as proxies for overall health and quality (Searcy and Yasukawa, 1996). Songbirds are even capable of recognizing individual conspecifics based on their vocalizations (e.g., Stoddard et al., 1992); for example, in some species, parents can recognize their offspring on the basis of distinctive begging calls (e.g., Stoddard and Beecher, 1983). Thus, perception of vocalizations serves many functions and is equally if not more important than song production for both male and female songbirds.

Given song's vitally important role in reproductive behaviour, it is not surprising that songbirds are experts at assessing particular aspects of song structure including amplitude (e.g., Searcy, 1996), duration (e.g., Weary et al., 1990), complexity (e.g., Clayton and Prove, 1989), and even note and syllable composition (e.g., Leitner and Catchpole, 2002). Numerous studies have demonstrated that songbirds are particularly good – better than humans – at classifying sounds based on absolute pitch (e.g., Friedrich, 2007, Weisman et al., 2010). It is thought that songbirds rely primarily on absolute pitch for auditory recognition (Hulse and Cynx, 1985; Cynx et al., 1986) and are not as good at tasks that rely on the use of relative pitch, such as the ratios or differences in pitch between notes (e.g., Page et al., 1989). Nonetheless, even if using absolute pitch is their strategy for pitch processing in the wild, songbirds are capable of performing relative pitch discriminations, as shown when they are presented with discrimination tasks that force them to use more than just absolute pitch (MacDougall-Shackleton and Hulse, 1996; Bregman et al., 2012).

Relative pitch processing takes on increased importance in songbirds with songs that vary in absolute frequency over a species-typical range, but maintain specific relative pitch ratios among the song's notes. In such cases, accurately detecting relative pitch can be crucial to conspecific recognition. For example, veeries (*Catharus fuscescens*) sing a simple, five-note song that varies considerably in absolute pitch but maintains a consistent pitch ratio between the third and fourth notes (Weary et al., 1991). In playback studies, males exhibited a stronger territorial response to songs with species-typical ratios between those notes compared to songs where that ratio was increased or decreased. (Weary et al., 1991). Likewise, the whistled notes of white-throated sparrow (*Zonotrichia albicolis*) song exhibit a ratio relationship that remains constant regardless of absolute pitch; like veeries, territorial males exhibit a weaker response when that ratio is altered (Hurly et al., 1992). These findings suggest that relative pitch processing plays an important role in species recognition in songbirds that feature such pitch ratio rules.

Similar to white-throated sparrows and veeries, black-capped chickadees (*Poecile atricapillus*) sing a simple two-note song that ranges in absolute frequency but changes little in terms of the pitch relationships within it. The chickadee's *fee bee* song is characterized by specific pitch ratios between the beginning and end of the *fee* note (start of *fee* / end of fee = 1.056) and between the end of the *fee* note and the beginning of the *bee* note (end of *fee* / start of *bee* = 1.134) (Weisman et al., 1990; See Figure 3.1). These pitch ratio "rules" are highly conserved throughout most of the bird's breeding range

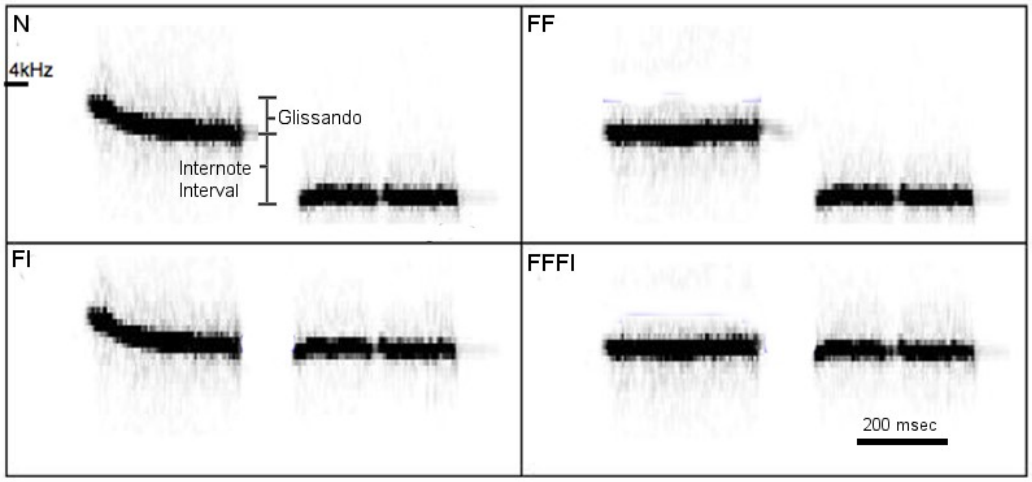

Figure 3.1 Spectrograms showing the stimuli used: Normal (N) (with species-typical pitch ratios labeled), Flat Fee (FF), Flat interval (FI), and Flat Fee / Flat interval (FFFI).

(Kroodsma et al., 1999). Black-capped chickadees are sensitive to – and employ – both absolute and relative pitch information. Lee et al. (2006) used an operant discrimination paradigm to show that black-capped chickadees are capable of sorting tones (ranging from 980-5660 Hz) based on absolute pitch, although they did a poor job at sorting tones in higher frequency ranges (above 5000 Hz). Recent research measuring auditory evoked potentials in response to tones ranging from 500-8000 Hz showed that chickadees are most sensitive to sound in the frequency range of their songs (3-4 kHz) (Wong and Gall, 2015). Field work indicates that chickadees employ absolute pitch in species recognition: birds responded less strongly when the absolute pitch of playback songs fell outside the typical range of chickadee song production (Weisman and Ratcliffe, 1989).

Operant research has demonstrated that chickadees are also capable of performing discriminations based on relative pitch. For example, Njegovan and Weisman (1997) tested male chickadees using an operant task in which the birds had to discriminate note pairs based on inter-note pitch ratio (the ratio between the frequency at the end of the first note and frequency at the start of the second). Males in the constant ratio group, in which responses to all stimuli with an approximately species-typical ratio (1.12) were rewarded, and responses to other stimuli (with ratios of 1.00 and 1.26) were not, successfully discriminated between the rewarding and non-rewarding stimuli. Males in the pseudo-ratio group, for whom the rewarding and non-rewarding stimuli were each a random mix of ratios, did not perform as well, demonstrating that the males in the constant ratio group actually employed relative pitch information in learning the task (Njegovan and Weisman, 1997). Hoeschele et al. (2012) found similar results using both male and female chickadees, as well as a larger array of inter-note pitch intervals (1.07, 1.13, 1.19, 1.25, and 1.31). In addition, they found that females performed better than males at discriminating the inter-note intervals.

Behavioural studies suggest that, like absolute pitch, the relative pitch relationships in chickadee songs are important for conspecific recognition and assessment. For example, territorial males respond less strongly to playback when the *glissando* within the *fee* note (i.e., the frequency change from the start to the end of *fee*) is altered but not when the inter-note pitch interval is changed, suggesting that males depend on the *glissando* to some degree for species recognition (Shackleton et al., 1992). In contrast, a lab study demonstrated that estradiol-implanted females show less sexual response to songs with either of the ratios altered, suggesting that they assess both ratios (Ratcliffe and Otter, 1996). Thus, these studies suggest sex differences in song perception, with female chickadees attending to both pitch ratios but males only attending to one of them.

In the previous chapter, I showed that both males and females respond vocally to playback when the inter-note pitch interval present, but not when it was absent. That is, the responsiveness of birds in our study appeared to depend on the presence of the inter-note interval, but not presence of the *glissando*. The inter-note interval may be particularly important in female mate choice: high-ranking males, which achieve higher reproductive success than lower-ranking ones (Otter et al., 1998), are better at reliably reproducing that particular species-typical ratio (Christie et al., 2004b). The fact that dominant males better maintain the inter-note interval raises the possibility that relative pitch cues within song may be important not just for conspecific recognition but for conveying information about the singer's quality to females and providing information for individual recognition of neighbours to males.

In this study, I wanted to compare directly whether male and female chickadees attend to one or both of the species typical ratios (the glissando and the inter-note interval) in the *fee bee* song. To do this, I used an operant training paradigm to test the abilities of black-capped chickadees to discriminate between songs containing the two species-typical pitch ratios and songs with one or both of those ratios altered. In light of the sex differences documented by previous behavioural work and a recent operant study (Hoeschele et al., 2012), I was particularly interested in possible sex differences in discriminative abilities. In addition, with previous behavioural data from our lab (Chapter 2) suggesting that both males and females attend to alteration of one species-typical pitch ratio (the inter-note interval) but not the other, I was curious to see whether birds from the same population would show differences in discrimination that mirror those response differences. Based on that previous work, I expected that the birds in this study would be better at discriminating between normal and altered songs when the inter-note interval was removed, compared to when the *glissando* within the *fee* note was flattened. Further, in keeping with recent operant research and evidence of the inter-note pitch interval's importance to mate choice, I expected that females would perform better than males on discriminations between songs with normal ratios and songs without an inter-note interval, but that males and females would not differ in performance while discriminating between songs with normal ratios and songs without a glissando.

3.2 METHODS

3.2.1 Animals and Housing

Eight male and nine female adult black-capped chickadees were captured within the Halifax Regional Municipality between February 2013 and May 2014 (CWS Permit # ST2779). Capture method depended on time of year. During the mid-winter, individuals were attracted using playback of chickadee vocalizations (calls and songs recorded in Ontario at least a decade before this study, as well as mobbing calls recorded at least 20 years before this study) and captured in potter traps baited with sunflower seeds. Later in the winter and during spring, when natural food supplies are abundant, individuals were attracted using the same playback recordings and captured using a mist net.

Sex and age were determined in the field using the same methods as in the previous study (Chapter 2), with non-adult birds released immediately. As in that study, sex and age were confirmed via examination of gonads and observation of skull pneumatisation, respectively, at the time of perfusion (after finishing the current study, birds were perfused for use in a separate immunohistochemistry study). Housing and details upon transport to the lab were the same as in the previous study (Chapter 2). All methods and procedures in this study were approved by Dalhousie University's University Committee on Laboratory Animals (Protocol # 12-023).

Birds captured during winter began operant conditioning once they were presumed to be in breeding condition, based on previous work demonstrating the effect of light exposure upon breeding status (Phillmore et al., 2005). I aimed to test all birds while they were in breeding condition in order to match the status of birds used in previous studies examining responses to normal and altered songs (e.g., Shackleton et al., 1992;

Ratcliffe and Otter, 1996). Thus, birds caught during mid-winter (e.g., February) were housed for 6-8 weeks under light cycles that reflected increasing natural day lengths prior to being put in operant chambers. Birds caught later (e.g., April and May) were kept in animal housing for only one day prior to being transferred to operant chambers because they were already in breeding condition.

3.2.2 Operant Apparatus

During the experiment, each bird lived and proceeded through the experiment within an operant chamber in a modified standard cage (37 x 31 x 24 cm deep) with a plastic grating floor attached to the bottom of the cage to ensure that spilled food was not accessible. Each cage was contained in a ventilated, sound-attenuating enclosure (65 x 46 x 42 cm deep) lighted with a 9-W twin-tube fluorescent bulb, and contained two perches, a water cup and a grit container. The perch in front of the feeder and the feeder itself were equipped with infrared beams used to monitor the location of the birds. The experiment was controlled by a microcomputer for each chamber and a desktop computer running Microsoft Windows XP for each pair of chambers. Song stimuli were stored on compact discs and played via disc drives within each desktop computer (one drive for each chamber). Stimuli were played via amplifier (Azur 350a, Cambridge Audio) to a speaker (Fostex FE108E) located inside the chamber beside the feeder and were broadcast at 70 dB SPL (measured from the perch using a sound level meter (Extech Instruments, model #407750).

3.2.3 Stimulus Vocalizations

Sets of stimulus vocalizations for discrimination were the same as those used in a previous study (Chapter 2) and consisted of *fee bee* songs of four different types: Normal (species-typical frequency ratios between the start and end of the *fee* note and between the end of the *fee* and start of the *bee*); Flat Fee (FF; frequency ratio from start to end of *fee* note reduced to 1.0); Flat interval (FI; frequency ratio between end of *fee* and start of *bee* reduced to 1.0); and Flat Fee/Flat interval (FFFI; both frequency ratio alterations) (Figure 3.1). I began with unaltered (Normal) recordings of six *fee bee* songs taken from the a set of recordings originally made between February 1999 and May 2000 that was used in previous work (Phillmore et al. 2003; 2011). These songs ranged in absolute frequency (measured at beginning of the bee note) from 3100-3350 Hz, increasing in 50 Hz increments, so as to reflect the normal range of *fee bee* song frequencies (Weisman et al., 1990); each stimulus began at a different frequency. Altered stimuli were prepared by eliminating one of or both of the frequency ratios in each of the original songs using the audio editing program Audition 2.0 (Adobe). This gave me six stimulus quads: one normal plus three altered stimuli made from each of the original *fee bee* recordings, giving me a total of 24 song stimuli to use for discrimination training and for probe testing.

Each stimulus set for non-differential and discrimination training comprised 5 of the 6 stimulus quads (i.e., 20 of the 24 recordings) described above, resulting in six different stimulus sets. Assignment of sets to the birds was done semi-randomly with the aim of using each one of the stimulus quads as generalization stimuli at least once. Birds were therefore trained with five normal, five Flat Fee, five Flat interval, and five Flat Fee Flat interval stimuli. Then, for probe testing, I added the remaining quad of stimuli to the stimulus pool to test whether the birds could generalize discrimination to a new, untrained, example of each stimulus type. For testing discrimination of Normal vs. FF songs in pseudo ratio group birds (see Procedure below), sets consisted of 12 song stimuli: all available Normal and FF stimuli.

3.2.4 Procedure

Preliminary training

Several stages of preliminary training were conducted in order to teach the bird how to use the booth and, eventually, respond after listening to each auditory stimulus. Each bird was first trained to use the perch and feeder in order to acquire food. Initially, the bird was familiarized with the location of food: the food cup remained in the up position and, at the beginning, additional food was scattered over the top of the food cup. During this stage, a red LED light behind the feeder was always on. Next, the bird was trained to activate the food cup and access food by flying into the feeder mechanism only when the red light was on. Following that, the bird was trained to sit on its perch for 1 second, across from the feeder mechanism, in order to activate the red light, at which point it could activate the food cup by entering the mechanism. The next stage was similar, but half the time the activated red light was accompanied by a tone: the bird was rewarded with food access for responding to the light combined with the tone, and punished with a 30-second intertrial interval (ITI) with the chamber lights out for responding to the light alone. In the final stage of feeder training, the role of the red light was removed and the bird was rewarded for responding to the tone and punished for responding when the tone was absent. Completion of each of the preceding two stages occurred when the bird discriminated the tone from no-tone contexts with a discrimination ratio (see Response Measures and Analysis below) of 0.90 for two consecutive days. The bird was then trained to remain on the perch until the end of the auditory stimulus before responding. In this stage, the bird again had to sit on the perch in order to activate a tone, upon which it was rewarded with food for flying to the feeder mechanism; unlike previous stages, the bird was punished for leaving its perch before the end of the tone with a 30-second ITI with the chamber lights out. This stage ensured that, during the following stages of non-differential and discrimination training, the bird would listen to the entire stimulus prior to responding.

Non-differential training

After learning to use the perch and feeder, each bird was exposed to the 20 unaltered and altered stimuli that it would hear during discrimination training, and trained to respond to all of them at a high and uniform rate. As in the preceding stages, the bird was required to sit on its perch for 1 second in order to activate the auditory stimulus. If the bird responded by entering the feeder mechanism during the 1 second following completion of the stimulus, it was rewarded with 1 second of food access. A 30-second ITI followed. If it left the perch but did not enter the feeder mechanism, the trial ended after 1 second. If the bird did not leave the perch, the trial ended after 1 second and a 60-second ITI followed; this was intended to encourage the bird to leave the perch after each stimulus. If the bird left the perch prior to completion of the stimulus, the trial ended and the chamber lights were turned off during a 30-second ITI; this ensured that the bird was listening to the entire auditory stimulus before responding. Stimuli were selected in random order without replacement until all were played, at which point all stimuli were returned to the pool for selection; birds trained continuously for the entire day and typically completed a total of about 1500 trials per day. Each bird remained in non-differential training until they were responding to all 20 stimuli at a rate of at least 70% and were leaving the perch prior to stimulus offset on less than 10% of trials.

Discrimination training

In discrimination training, some of the stimuli were rewarded (S+) while some were not (S-). In the constant ratio group (6 males and 6 females), the five normal song stimuli in a particular bird's stimulus set were S+s, while the 15 altered song stimuli (five each of FF, FI, and FFFI) were S-s. In the pseudo ratio group (3 males and 2 females), 5 of the 20 stimuli were randomly selected to be S+s in the case of each bird. Entering the feeder following an S+ stimulus was rewarded with 1 second of food access. Entering the feeder after an S- stimulus was punished with a 30-second ITI with the chamber lights out, therefore the correct response after hearing an S- was to leave the request perch and then return to start a new trial. The contingencies for other scenarios that were in place during non-differential training (e.g. zaps) also applied during discrimination training. Stimuli were selected in random order without replacement until all stimuli were played, at which point all stimuli were returned to the stimulus pool and the process repeated itself. In total, birds typically completed about 1500 trials per day. Discrimination training continued either for 15 days or until birds achieved a discrimination ratio of 0.90 over three consecutive 500-trial blocks.

Probe testing

For the constant ratio group, one day of probe training was conducted in order to ensure that each bird was discriminating based on the use of pitch-related rules rather than memorization. Probe testing was preceded by two days of modified discrimination training, during which S+ stimuli were rewarded on 85% of correct visits to the feeder rather than 100% in order to reduce each bird's ability to discriminate probe stimuli from training stimuli based on how often they were rewarded. During probe training, stimuli included the same S+ and S- stimuli as in training, as well as the stimulus group (one each of Normal, FF, FI, and FFFI) not initially included in each particular bird's training set. While feeder visits to the S+ stimuli from discrimination training were again rewarded 85% of the time, all four of the new stimuli were rewarded on 15% of correct visits. While having these new stimuli rewarded occasionally encouraged birds to continue responding, the low and uniform rate at which they were rewarded assured me that bird's responses to the probe stimuli were due to transfer of rules learned during discrimination training rather than new learning during probe testing. The contingencies present during discrimination training remained in place here. The 24 stimuli were selected and played in random order without replacement. Birds typically completed 1000-2000 trials during probe testing; the first 1000 trials of each bird were used in analyses.

Pseudo ratio group normal versus flat fee training

In order to further explore an interesting trend that emerged in the constant ratio group's data, birds in the pseudo ratio group underwent an additional round of discrimination training. In it, birds were presented with 12 stimuli: six normal (S+) and six FF (S-). I did this to force the birds to attend to the FF stimuli in the discrimination to determine if they were simply ignoring them when there were other S- types, or if only the lack of glissando made the discrimination particularly difficult. The contingencies present in the previously described discrimination training were also in place here. Stimuli were again selected in random order without replacement. I used the pseudo ratio group for this because birds from that group had not received training consistent with stimulus category, yet were already familiar with the stimuli and the apparatus.

3.2.5 Response Measures and Analyses

Data were collected in blocks of 500 trials, but for analyses, trials were combined into bins of 1000 trials. For discrimination training, an overall discrimination ratio was calculated for each 1000-trial bin by dividing the number of feeder visits in response to S+ stimuli by the total number of visits in response to both S+ and S- stimuli. Thus, a discrimination ratio of 0.50 would represent birds responding equally to S+ and S- stimuli (i.e., responding at chance levels and not discriminating), while a ratio of 1.00 would indicate perfect discrimination (responding only to S+ stimuli). Individual discrimination ratios were also calculated with respect to each type of altered (S-) stimulus (FF, FI, and FFFI); for example, the discrimination ratio for FFFI stimuli was calculated by dividing the number of feeder visits in response to S+ stimuli by the total number of feeder visits to both S+ and FFFI S- stimuli.

For each bin, I also calculated how many of the five S+ stimuli were being successfully discriminated from the S- stimuli in order to make sure that birds were attending to and learning all S+ stimuli rather than generating a high discrimination ratio by responding to only one or two S+ stimuli. To do this, I calculated the response rate (number of visits to the feeder divided by the total number of times leaving the perch) for each stimulus and then used those numbers to determine how many of the S+ stimuli had a response rate higher than the upper bound of the 95% confidence interval (i.e., mean response rate plus two standard deviations) of the response rates for the S- stimuli. As with discrimination ratio, this was calculated both overall and individually with respect to each type of altered stimulus (FF, FI, and FFFI).

Both discrimination ratio and the number of S+ stimuli successfully discriminated were calculated for each of 16 bins (16,000 trials), as well as the final bin during discrimination training. The final bin for each bird represented the point at which they achieved criterion (three consecutive 500-trial blocks with an overall discrimination ratio of at least 0.90) or, if that criterion was not reached, the last bin completed during discrimination training. In the case of each measure, data were used to conduct repeated measures analyses of variance (ANOVAs) in order to determine whether birds in the constant ratio group discriminated more successfully than those in the pseudo ratio group. ANOVAs were also conducted to look for differences in discrimination of the different types of altered stimuli (FF, FI, and FFFI), as well as for possible sex differences.

All statistical analyses were conducted in IBM SPSS Statistics 22 (Windows). Discrimination data was assessed in terms of 1000-trial bins. When data failed Mauchly’s Test of Sphericity, a Greenhouse-Geisser (GG) correction was applied to ANOVA results.

3.3 RESULTS

3.3.1 Constant Versus Pseudo Ratio Discrimination

Discrimination ratio

Among the birds in the constant ratio group, the criterion of three consecutive days with a DR of at least 0.90 was reached by two of six males (reached after 23 and 28 1000-trial bins) and three of the six females (after 18, 23, and 24 bins); the birds that did not reach the 0.90 criterion completed an average of 25.71 (SD = 2.15) bins after 15 days of training. None of the birds in the pseudo ratio group (n = 5) reached the criterion; they completed an average of 26.17 (SD = 12.24) bins after 15 days of training.

Comparison of overall discrimination ratios revealed that, while discrimination of rewarded versus unrewarded stimuli improved over the course of training in both the constant ratio and pseudo ratio groups, performance was better in the constant ratio group (Figure 3.2a). A sex x ratio group x bin mixed ANOVA showed a main effect of bin (F(16,208) = 39.772, $p < 0.001$), a main effect of ratio group (F(1,13) = 16.088, p = 0.001), but no main effect of sex. A significant bin x ratio group interaction (F(16,208) = 1.804, p = 0.032) led me to conduct separate one-way ANOVAs on bins for each ratio group.

For each ratio group there was a main effect of bin (constant: F(16,176) = 72.415, $p < 0.001$; pseudo: F(16,64) = 6.168, $p < 0.001$), reflecting improving discrimination over the course of training within each ratio group. Post-hoc comparisons of discrimination ratios between consecutive bins revealed that birds in the constant ratio group began improving immediately: their discrimination ratios improved significantly from one bin to the next over the first five bins; performance also improved from bins 6 to 7 and between bin 16 and the final bin. In contrast, the pseudo ratio group only showed such bin-to-bin improvements between bins 9 and 10 and bins 14 and 15. Independent t-tests to compare performance between the constant versus pseudo ratio groups at each bin revealed that discrimination ratios were higher in the constant ratio group compared to the pseudo ratio group ($p < 0.05$) at bins 3-5, 10-12, and 14; final discrimination ratios were also higher in the constant ratio group than the pseudo group.

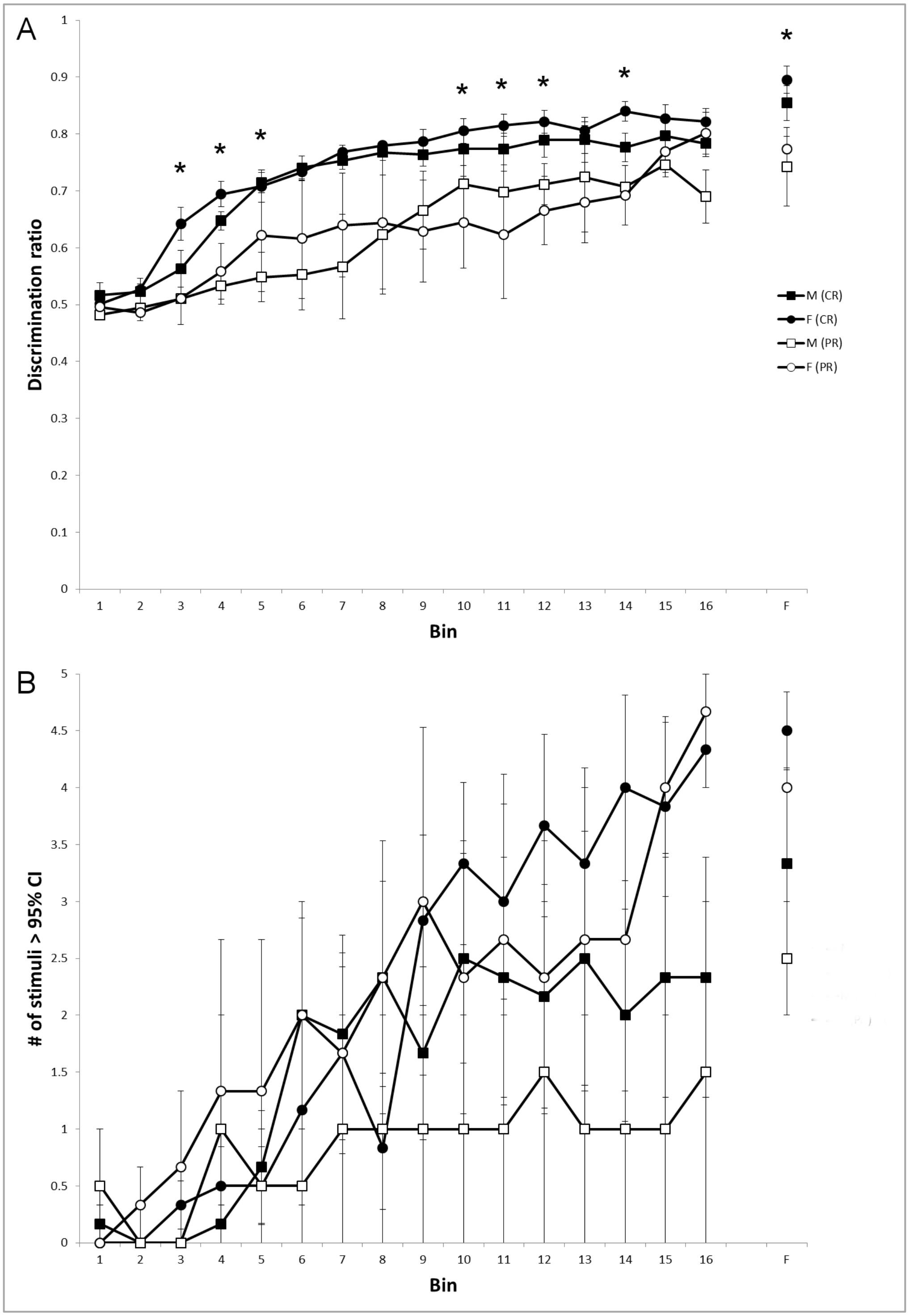

Figure 3.2 Discrimination ratio (A) and number of S+ stimuli successfully discriminated (B) by constant ratio and pseudo ratio group birds during discrimination training. Error bars represent standard errors of the means. Asterisks (*) indicate bins in which performance of constant ratio and pseudo ratio groups was significantly different ($p < 0.05$).

Confidence intervals

The number of S+ stimuli successfully discriminated from S- stimuli (see Methods for details on calculation) increased over the course of training for both the constant ratio and pseudo ratio groups, although there was no overall difference between the groups in terms of performance (Figure 3.2b). A sex x ratio group x bin mixed ANOVA revealed a main effect of bin ($F(16, 208) = 11.363$, $p < 0.001$) but no main effects of sex or ratio group. However, there was a bin x sex interaction ($F(16,208) = 1.720$, $p = 0.045$), which prompted me to conduct separate one-way ANOVAs on bins for each sex. For both males and females, the ANOVAs revealed main effects of bin (male: $F(16,112) = 5.430$, $p < 0.001$); female: $F(16,128) = 13.942$, $p < 0.001$). To further explore the interaction, I compared males and females for each bin during discrimination training. Independent t-tests comparing male and female performance revealed that females performed better than males at the end of discrimination training (Bin 16).

Overall, discrimination ratio data indicated that birds in the constant ratio group learned to discriminate S+ from S- stimuli more quickly than birds in the pseudo ratio group. Data related to the number of stimuli successfully discriminated was much more variable and therefore did not reveal a significant difference between birds in the two groups. There was, however, a sex difference: females ended up learning to accurately discriminate more of the S+ stimuli (about four of the five) than males (about three of the five).

3.3.2 Discrimination of Normal Versus Altered *Fee Bee* Songs

Discrimination ratio

Within the constant ratio group, the ability to discriminate between normal songs (S+) and each type of altered (S-) stimulus (FF, FI, and FFFI) was assessed by calculating separate discrimination ratios for each S- type. Comparison of these discrimination ratios revealed that performance differed depending on the type of S- stimuli, with birds having the greatest difficulty performing the discriminations when FF was the S- stimulus (Figures 3.3a and 3.3b). Discrimination ratios for each type of S- were compared using a sex x stimulus type x bin mixed ANOVA, which revealed a main effect of stimulus type

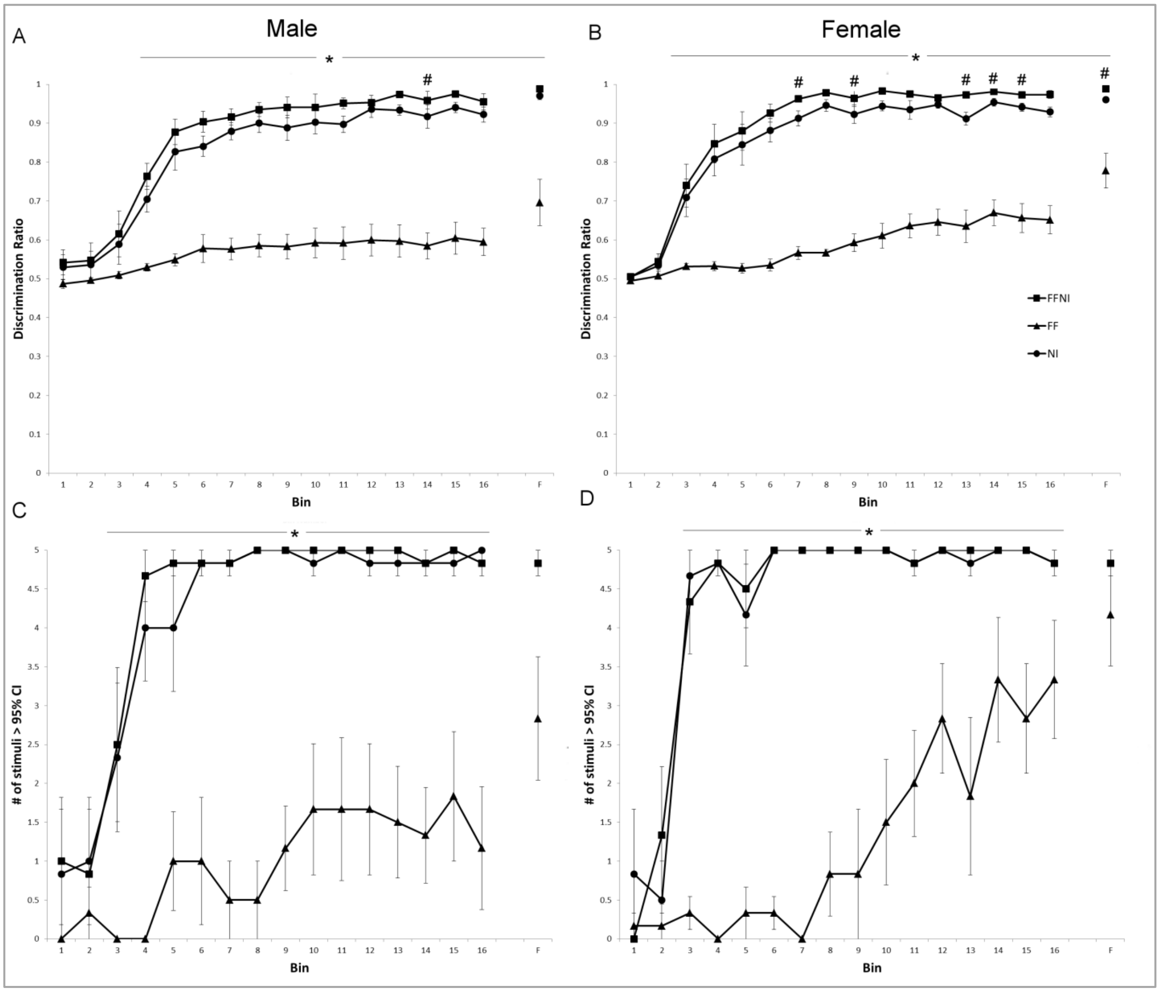

Figure 3.3 Discrimination ratios (A-B) and number of stimuli successfully discriminated (C-D) of constant ratio group birds during discrimination training, broken down by type of altered stimulus. Error bars represent standard errors of the means. Asterisks (*) indicate bins in which discrimination of FI and FFFI differed significantly from FF, # indicates bins in which discrimination of FI differed significantly from FFFI ($p < 0.05$).

(GG-corrected F(1.076,10.759) = 156.300, $p < 0.001$) and bin (F(16,160) = 95.930, $p < 0.001$). There was a significant interaction between stimulus type and bin (GG-corrected F(3.134,31.343) = 32.745, $p < 0.001$) and among sex, stimulus type, and bin (GG-corrected F(4.144,41.445) = 2.469, $p < 0.001$), which prompted me to conduct a stimulus type x bin mixed ANOVA separately for each sex.

For each sex, there was a significant main effect of stimulus type (male: GG-corrected F(1.036,5.178) = 54.237, $p = 0.001$; female: F(2,10) = 137.303, $p < 0.001$) and bin (male: F(16,80) = 44.861, $p < 0.001$; female: F(16,80) = 52.657, $p < 0.001$). There was also a significant interaction between stimulus type and bin (male: GG-corrected F(3.799,18.996) = 20.063, $p < 0.001$; female: F(32,160) = 15.766, $p < 0.001$). These interactions were further explored using bin-by-bin one-way ANOVAs comparing stimulus types within each sex. An overall effect of stimulus type appeared earlier in females (Bin 3) than in males (Bin 4), and persisted over the remainder of training for each sex. Bonferroni-corrected post-hoc comparisons revealed that females discriminated better when FFFI was the S- than when FI was the S- earlier and more frequently (bins 7, 9, 13, 14, 15, and final) than did males (bin 14) ($p < 0.05$).

Only one of the birds, a female, reached the criterion of three consecutive days with a DR of at least 0.90 with respect to discrimination of Normal S+ from FF S- stimuli; it did so after 38 bins, well after it had reached overall criterion. The number of trials required to reach criterion with respect to FI and FFFI S- stimuli is shown in Figure 3.4. A sex x stimulus type ANOVA of trials to criterion for FI and FFFI stimuli revealed a main effect of stimulus type ($F(1,10) = 5.498$, $p = 0.041$), but no main effect of sex or no interaction.

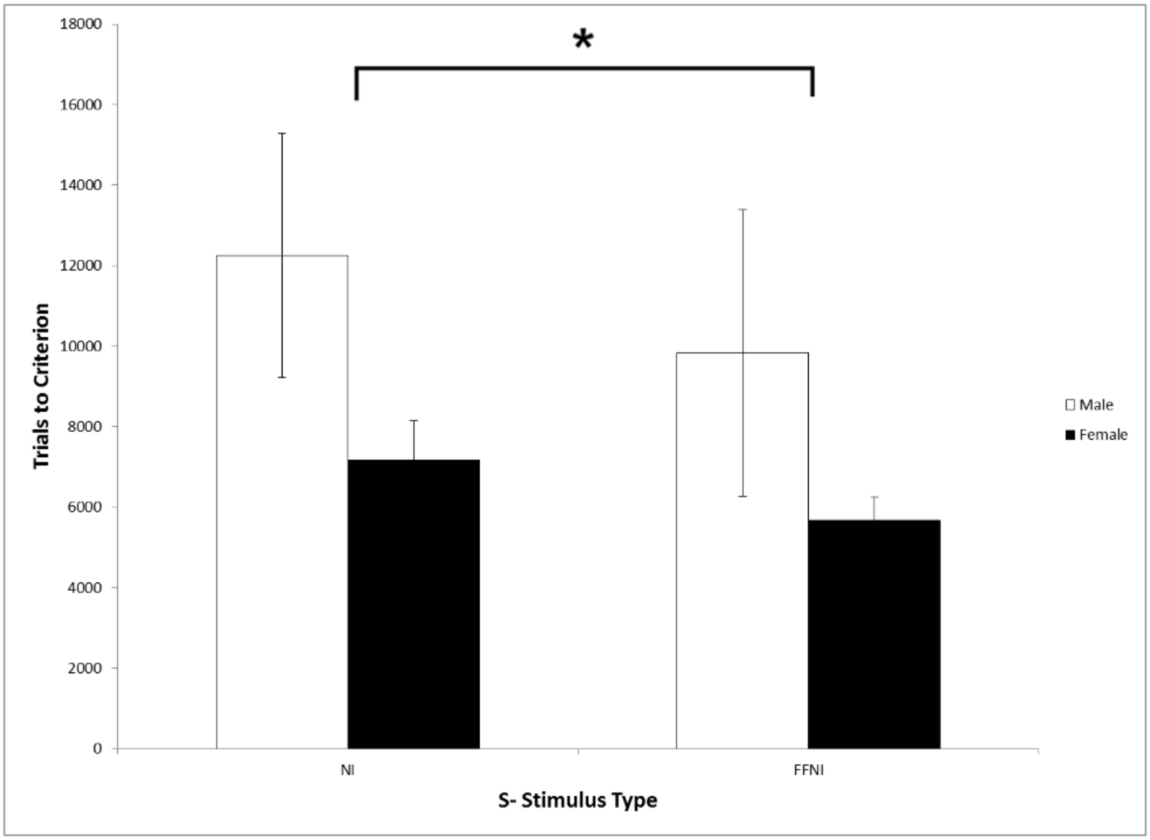

Figure 3.4 Number of trials required to reach the criterion of three consecutive 500-trial bins with a discrimination ratio of at least 0.90, with respect to FI and FFFI S- stimuli. Asterisks (*) indicate $p < 0.05$.

A paired t-test comparing trials to criterion for these two stimuli revealed that birds reached criterion quicker with respect to FFFI S- stimuli than for FI S- stimuli ($p = 0.034$).

Confidence intervals

As with discrimination ratio, the number of S+ stimuli that were successfully discriminated differed across the different types of S- stimuli, with the fewest S+ stimuli discriminated with respect to FF S- stimuli (Figures 3.3c and 3.3d). A sex x stimulus type x bin mixed ANOVA revealed main effects of bin ($F(16,160) = 34.227$, $p < 0.001$) and of stimulus type (GG-corrected $F(1.019,10.194) = 89.255$, $p < 0.001$), with no main effect of sex or interactions involving sex. There was a significant interaction between stimulus type and bin (GG-corrected $F(4.963,49.627) = 8.253$, $p < 0.001$), which prompted me to examine differences in S+ learning across S- stimulus types using one-way ANOVAs to compare S- stimulus types at each bin. The number of S+s learned did not differ based on S- type until bin 3, but this difference continued over the remainder of the training bins (including final); over most of training, birds learned different numbers of S+s depending on the type of S- stimulus. Bonferroni-adjusted post-hoc comparisons by stimulus type revealed that, from bin 3 through bin 16, birds could discriminate more S+s from S-s when either FFFI or FI was the S- than when FF was the S-. For the final bin, there were no differences between any particular pairs of S- types despite the overall effect of stimulus type.

3.3.3 Discrimination of Normal Versus Flat Fee (FF) Songs

To further investigate the relatively poor discrimination of normal versus FF songs during discrimination training by birds in the constant ratio group, I ran birds in the pseudo ratio group through a second training period, following primary discrimination training, in which they had to discriminate exclusively between normal (S+) and FF (S-) song stimuli (i.e., there were no FI or FFFI song stimuli during these trials). Comparison of performance by the pseudo ratio group birds during this secondary training to discrimination of normal songs from the FF S- stimuli by the constant ratio group birds during discrimination training revealed no difference in discrimination of normal from FF songs (Figure 3.5). A group (constant vs. pseudo) x sex x bin (6 bins plus final performance) mixed ANOVA revealed a main effect of bin (GG-corrected F(1.457,18.938) = 23.347, $p < 0.001$), but no main effects of sex or group. There were no interactions involving the group factor, indicating birds with only FF as S- stimuli did not perform any better than birds with all S- stimuli. There was a significant interaction between sex and bin (GG-corrected F(1.457,18.938) = 4.621, $p = 0.033$), which prompted me to run separate ANOVAs for each sex. These revealed main effects of bin for both sexes (male: GG-corrected F(1.510,10.570) = 8.954, $p = 0.008$; female: GG-corrected F(1.312,10.492) = 33.438, $p < 0.001$). Bonferroni-corrected post-hoc comparisons of performance, with the two ratio groups pooled together, revealed that males did not show significant improvement from any one bin to the next, while for females there was

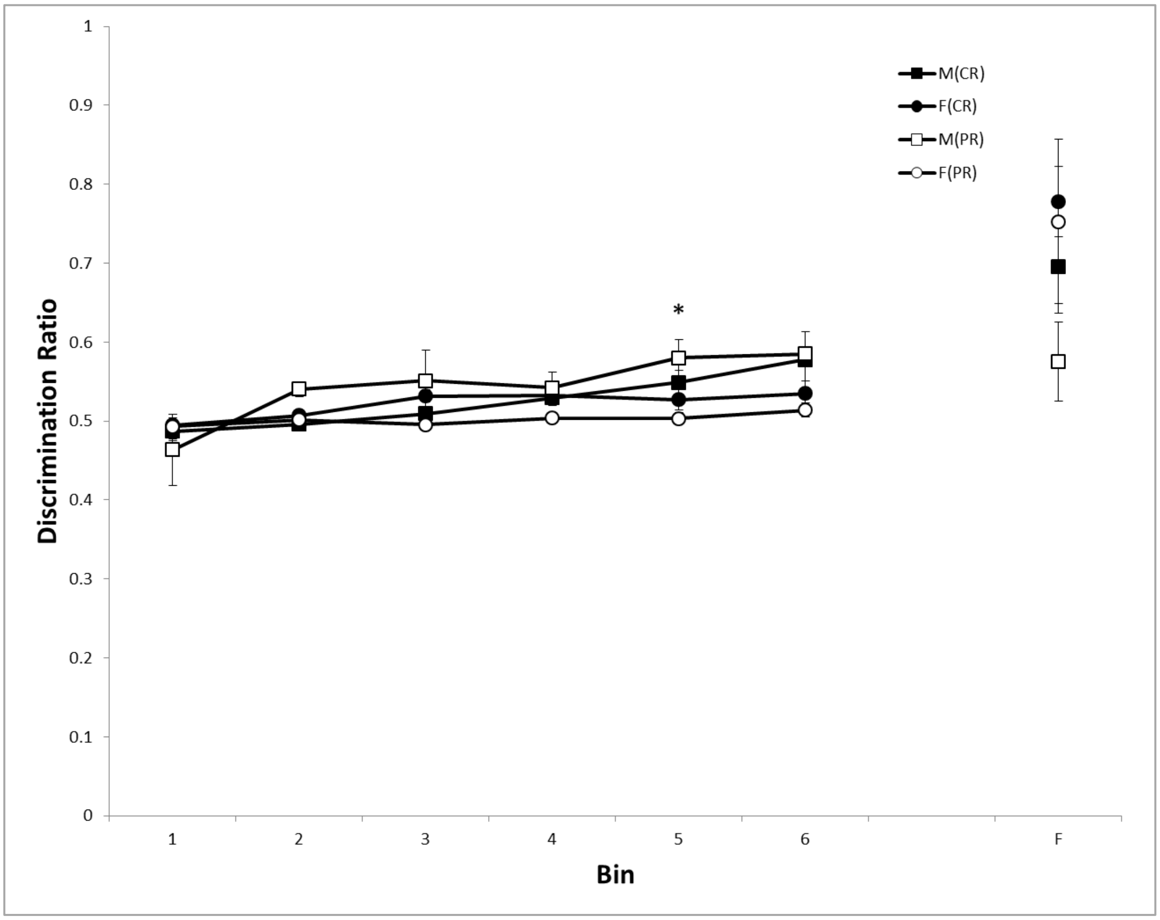

Figure 3.5 Discrimination of Normal versus FF stimuli by constant ratio group birds during discrimination training and pseudo ratio group birds during a second training period featuring exclusively Normal and FF stimuli. Error bars represent standard errors of the means. Asterisks (*) indicate a significant difference between males and females ($p < 0.05$).

significant improvement between bin 6 and the final bin. Independent t-tests for each bin revealed that males and females discriminated between Normal S+ and FF S- stimuli at similar levels except in bin 5, where males performed better ($p < 0.05$). Overall, there was no difference between the groups with respect to discrimination of normal versus FF song stimuli.

3.3.4 Probe Testing

In order to determine whether the category learning (normal vs. altered) by the constant ratio group was transferrable to new stimuli, I examined whether birds treated new S+ and S- stimuli introduced during probe testing as they did the S+ and S- stimuli from discrimination training. To do this, I calculated response rates to the novel S+ and S- stimuli and compared these to the response rates to pre-existing S+ and S- that were presented during that same probe test. If learning was transferred to new stimuli, response rates to new S+ and S- stimuli should be high and low, respectively, similar to the already trained stimuli. That was the case, as birds responded at similar rates to each type of newly introduced stimuli as they did to pre-existing stimuli; that is, the responses to new S+ and S- stimuli were similar to responses to old S+ and S- stimuli, respectively (Figure 3.6). A sex x training phase (training vs. probe) x reward (S+ vs. S-) mixed ANOVA revealed a significant main effect of training phase ($F(1,10) = 8.041$, $p = 0.018$), with post-hoc comparisons revealing that birds responded more to trained stimuli than to probe stimuli. There was also a significant main effect of reward ($F(1,10) = 246.058$, $p < 0.001$), with birds responding more to trained and probe S+ stimuli than to trained and probe S- stimuli. There was a significant interaction between phase and reward ($F(1,10) = 12.804$, $p = 0.005$): birds responded more to trained S+s than to probe S+s, but there was no difference in responding between trained and probe S-s. In other words, birds transferred their training to previously untrained stimuli, although responding was not as strong to S+ probe stimuli as to training stimuli. In summary, probe testing indicated that, during discrimination training, birds were applying pitch ratio rules rather than merely memorizing which stimuli were rewarding (S+).

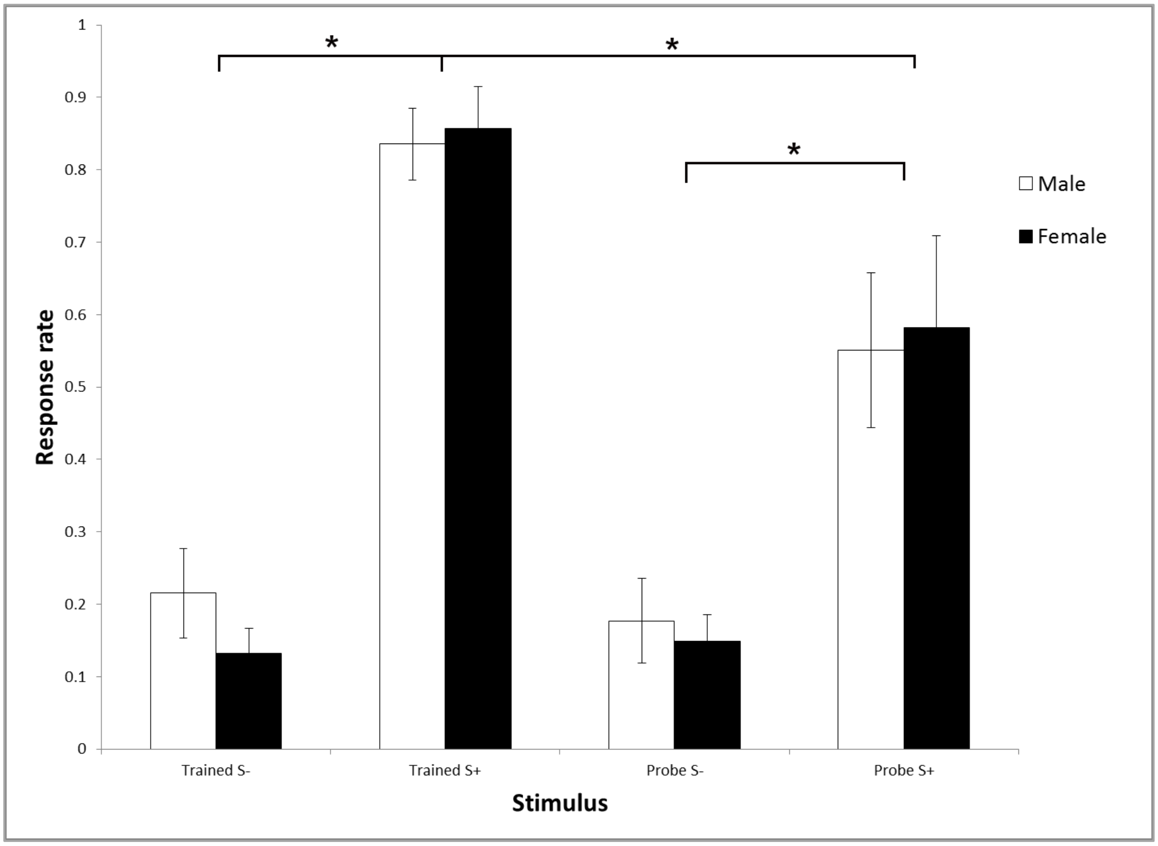

Figure 3.6 Response rates during probe testing of male and female birds to previously trained S+ and S- and S+ and S- stimuli newly introduced during probe testing. Error bars represent standard errors of the means. Asterisks (*) indicate $p < 0.05$.

3.3.5 Discrimination at Different Absolute Frequencies

The song stimuli used in this study consisted of six sets (each containing a normal stimulus and three altered stimuli (FF, FI, and FFFI)) that ranged in absolute pitch from 3100 to 3350 Hz (as measured at the start of the *bee* note). Comparison of discrimination ratios by stimulus frequency provided evidence that performance differed based on absolute pitch. In keeping with earlier analyses, a pitch x sex x stimulus type x bin mixed ANOVA revealed main effects of bin ($F(16,160) = 129.442$, $p < 0.001$) and stimulus type (GG-corrected $F(1.079,10.793) = 230.206$, $p < 0.001$) but no main effect of sex. While there was no main effect of pitch, a significant interaction between pitch and stimulus type (GG-corrected $F(3.609, 36.090) = 8.357$, $p < 0.001$) suggested that the relationship between discrimination and absolute pitch varied with stimulus type. Thus, I conducted separate ANOVAs for each stimulus type.

During training, discrimination of normal versus FF stimuli differed depending on the absolute pitch of the stimuli (Figure 3.7a). In addition to the expected main effect of bin ($F(16,160) = 26.830$, $p < 0.001$), a sex x pitch x bin mixed ANOVA of FF discrimination ratio data revealed a main effect of pitch ($F(5,50) = 5.700$, $p < 0.001$). There were also significant interactions between sex and bin ($F(16, 160) = 1.979$ $p = 0.017$) and between pitch and bin ($F(80,800) = 1.585$, $p = 0.001$). In light of the pitch x bin interaction, one-way ANOVAs were conducted for each bin in order to investigate the above-noted main effect of pitch. There were main effects of pitch in bins 4 and 5, 7 through 11, 14 and 15. Post-hoc comparisons by pitch suggest that birds discriminated Normal from FF stimuli better at the highest frequencies. For example, discrimination of 3350 Hz stimuli was higher than all other stimuli in bin 4; similarly, discrimination of 3300 and 3350 Hz stimuli was higher than the other stimuli in bins 9 and 10.

As with FF stimuli, discrimination of normal versus FI stimuli also differed with absolute pitch of the stimuli (Figure 3.7b). The same three-way repeated measures ANOVA was conducted for FI discrimination ratio data. In addition to the expected main effect of bin ($F(16,160) = 117.728$, $p < 0.001$), there was a main effect of pitch (GG-corrected $F(2.784,27.842) = 5.137$, $p = 0.007$) and a significant interaction between pitch and bin ($F(80, 800) = 1.729$, $p < 0.001$). There was no effect of sex. To explore differences related to absolute pitch over the training period, one-way ANOVAs were conducted for each bin. There was an overall effect of pitch in bins 4, with 3250 Hz stimuli discriminated better than 3300 and 3350 Hz stimuli. This overall effect was also present in bins 6-8, with 3250 Hz stimuli discriminated better than all but 3200 Hz stimuli in bin 6, better than all but 3350 Hz stimuli in bin 7, and better than all other stimuli in bin 8. Overall, 3250 Hz stimuli were learned more quickly than those of other pitches.

Unlike the other types of S- stimuli, discrimination of normal versus FFFI stimuli did not differ on the basis of absolute pitch (Figure 3.7c). A pitch x sex x bin repeated measures ANOVA of FFFI discrimination ratio data revealed the expected main effect of bin ($F(16,160) = 137.825$, $p < 0.001$), but no main effect of pitch or sex. However, there was a significant interaction between pitch and bin ($F(80,800) = 1.357$, $p = 0.025$), which prompted me to examine performance at each bin using a series of one-way ANOVAs.

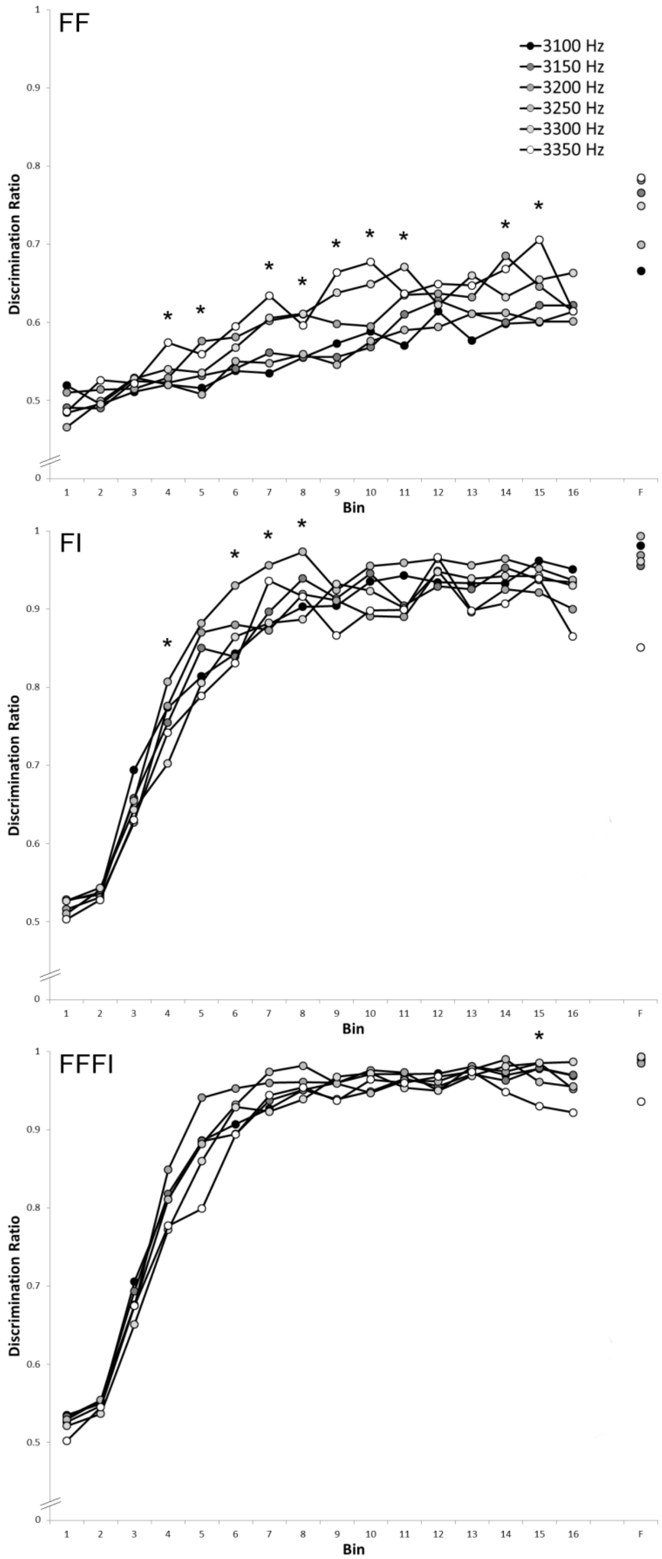

Figure 3.7 Discrimination of Normal versus each S- stimulus type, broken down by absolute stimulus frequency. Error bars represent standard errors of the means. * indicates an overall effect of absolute frequency.

Only bin 15 displayed a main effect of pitch, with post-hoc comparisons revealing that stimuli of the highest frequency (3350 Hz) were discriminated more poorly than 3150, 3200 and 3300 Hz stimuli.

Overall, analysis of discrimination data by absolute pitch suggests that birds learned to discriminate S+ stimuli from altered S- stimuli more quickly at high frequencies when the S- stimuli were FF and at mid-range frequencies when the S- stimuli were FI. No comparable pattern emerged for discrimination of Normal from FFFI S- stimuli.

3.4 DISCUSSION

The main objective of this study was to examine whether male and female black-capped chickadees could discriminate between normal *fee bee* songs and songs with altered species-typical pitch ratios associated with species recognition. Birds relied on the pitch ratio rules rather than individual memorization to categorize stimuli: the constant ratio group (where normal songs were rewarding and altered ones were non-rewarding) learned more quickly and achieved higher overall accuracy than the pseudo ratio group (where rewarding and non-rewarding stimuli were each a mix of normal and altered songs). In addition, probe testing in the constant ratio group indicated that birds were able to transfer their learning: they exhibited stronger responses to normal than to altered songs not just with familiar stimuli but also with novel stimuli of a different absolute frequency not presented during discrimination training.

Although chickadees learned to discriminate between songs with typical ratios and songs with altered ratios, some alterations were easier to discriminate than others. Birds quickly learned to discriminate between normal songs and those with the inter-note interval removed (FI: frequency ratio from end of *fee* to start of *bee* reduced to 1.00), matching previous operant study findings showing that chickadees can discriminate between songs that differ with respect this ratio (Njegovan and Weisman, 1997; Hoeschele et al., 2012). They did similarly well – and, in some cases, better – at discriminating normal songs from songs with both the inter-note interval and the *glissando* removed (i.e., ratio from start to end of *fee* note reduced to 1.00) (FFFI). However, most birds had difficulty learning to discriminate normal songs from those with only the *glissando* eliminated (FF): final discrimination ratios for normal versus FF did not exceed 80%, and most birds never met overall criterion (three days with a discrimination ratio over 0.90). A subset of chickadees forced to discriminate between only normal and FF songs exhibited the same difficulty in learning this discrimination.

These findings contrast with those of previous behavioural studies suggesting that males require the *glissando* but not the inter-note interval for conspecific recognition, while females attend to both the glissando and inter-note interval (Shackleton et al., 1992; Ratcliffe and Otter, 1996). The poor performance of birds of both sexes in this study with respect to discrimination of the *glissando* may have resulted from the birds focusing more on the inter-note interval discrimination, possibly due to greater biological relevance, at the expense of the *glissando.* Given a choice between using the inter note interval and the glissando, in the context of this study, birds may have focused on the feature of the *fee bee* that was perhaps easiest to identify as absent; the missing glissando may be a much less discriminable feature, especially compared to having consecutive notes end/begin on the same frequency. The similar performance of the birds forced to attend to the *glissando* suggests that the results may instead reflect poor perceptual abilities, although it is possible that their performance was influenced by the preceding discrimination training, which featured random assignment of S+ stimuli and therefore relied on a different strategy, memorization.

Recent work in our lab, using the same normal and altered song stimuli as this study within a different experimental setup, found that vocal response (number of vocalizations to playback) did not differ between normal and FF stimuli, but were lower in response to songs missing the inter-note interval (FI and FFFI) (Chapter 2). These results mirror the differences in discriminative performance seen in the current study in that birds did not seem to recognize when the glissando was missing, but did recognize the absence of the inter-note interval.

As detailed in the previous chapter, differences between findings in our lab and those of previous studies may stem from a variety of factors, including geographic variation in how perception of and attendance to different aspects of song structure among black-capped chickadees. The effects of captivity and lab experimentation may also have influenced behaviour, as has been documented in other songbirds (e.g., Dickens and Bentley, 2014). Even the foundational behavioural studies examining attendance to the species-typical pitch ratios (Shackleton et al., 1992; Ratcliffe and Otter, 1996) did not compare sexes directly, used different response measures, and were carried out in different venues (field versus laboratory).

In some respects, female chickadees outperformed males on our operant task. Females improved more quickly at discriminating normal from altered stimuli during the early stages of discrimination training, showing their first significant bin-to-bin improvement one bin (1000 trials) earlier in training than males. Females also performed better at the end of discrimination training: they learned to discriminate approximately one more of the five S+s used in training than did males. These findings partly mirror those of Hoeschele et al. (2012); females performed better than males at the end of a similar discrimination task. Although males in that study performed better than at earlier stages of discrimination training, they did so only when discriminating among three species-atypical ratios (ratio of S+: 1.25; ratios of S-: 1.19 and 1.31); in this study, the rewarded (S+) stimuli within the constant ratio group always featured the species-typical ratio.

It may be that females have evolved better discriminative abilities in relation to the inter-note pitch interval due to that ratio's importance in assessing male quality. Both dominant males and the females that mate with them experience greater reproductive success (Otter et al., 1998; Doucet et al., 2005), and dominant males are better than non-dominant ones at maintaining the species-typical inter-note interval (Christie et al., 2004b). If being able to accurately detect changes in this ratio is an important contributor to mate selection and eventual reproductive success, it may be that females have been forced to develop more finely tuned discriminative abilities. Linking discriminative ability to breeding condition would be the next logical step in this research. Females implanted with estradiol, mimicking the levels found while in breeding condition, have more finely tuned selectivity in neural response in perceptual regions of the brain than birds without estradiol (Maney and Pinaud, 2011). Therefore comparing performance of females in breeding condition to those in non-breeding condition, and in turn to males, may clarify the degree to which females use inter-note interval in mate selection, and whether their ability to assess the ratio is enhanced in comparison with males.

Besides the recent findings by Hoeschele et al. (2012), there is little information available about how male and female chickadees compare with regard to relative pitch processing. In terms of absolute pitch processing, Lee et al. (2006) found no differences between male and female chickadees on an operant pitch-sorting task. Wong and Gall (2015), examining auditory sensitivity at different absolute frequencies by measuring auditory evoked potentials in response to tonal sound stimuli, also detected no sex differences in black-capped chickadees. However, sex differences in pitch processing have been detected in Carolina chickadees (*Poecile carolinensis*), a closely related species with a similar vocal repertoire: Henry and Lucas (2010), measuring auditory brainstem responses, found that frequency selectivity (the resolution with which a bird can differentiate between frequencies) was greater in females than males. As Wong and Gall (2015) suggest, examination of frequency resolution in black-capped chickadees may reveal sex differences similar to those seen in Carolina Chickadees.

Lastly, there was evidence that discriminative performance differed depending on absolute frequency. The experiment featured six sets of song stimuli, increasing in 50 Hz increments between 3100 and 3350 Hz (measured at the start of the *bee* note), spanning the middle portion of the species' natural range of song production (2770-3630 Hz; Christie et al., 2004b); each bird was exposed to five of the six sets during discrimination training, allowing for comparison of performance across absolute song frequencies. Birds learned to discriminate normal from FI (inter-note interval = 1.00) quicker for the stimuli at the intermediate frequency of 3250 Hz than for those at higher or lower frequencies. In contrast, discrimination of normal versus FF (pitch ratio across *fee* = 1.00) stimuli began improving earlier at the highest frequency, 3350 Hz, compared to the others.

There is little known about how relative pitch processing varies with absolute frequency in black-capped chickadees and other songbird species. However, an operant discrimination study by Weary and Weisman (1991) provides some support that certain frequencies are better discriminated than others by black-capped chickadees. In probe trials conducted after the birds had learned to discriminate between songs with normal (rewarding) and altered (non-rewarding) inter-note pitch intervals, subjects responded more strongly (i.e., made more feeder visits) to songs with species-typical pitch ratios than to those with atypical ratios, but only at intermediate absolute frequencies. While the optimal discrimination in their study occurred at lower frequencies (~ 3050 Hz) than those used in our study (3100-3350 Hz), Weary and Weisman (1991) nonetheless demonstrate that assessment of relative pitch processing may vary with absolute pitch in black-capped chickadees. The differences in optimal discrimination ranges between studies may relate in part to the fact that I used songs recorded in the wild and then manipulated, while they used completely synthesized stimuli (Weary and Weisman, 1991), a difference which may influence performance on operant discrimination tasks (Hoeschele et al., 2012). It is also possible that these differences related to geographic variation in song perception, as discussed in the previous chapter.

Across songbirds, there is extensive evidence of coevolution between vocal production and auditory processing abilities (e.g., Woolley and Moore, 2011). Such signal-perception coevolution has been shown in black-capped chickadees with respect to auditory sensitivity (Wong and Gall, 2015), and may be a factor in other aspects of auditory perception, such as relative pitch processing. In support of this, recent evidence from sparrows suggests that not only frequency but also the structure of vocalizations may influence auditory sensitivity (Velez et al., 2014). Especially in species where relative pitch cues appear to carry important information (e.g., about individual identity and male quality in black-capped chickadees; Christie et al., 2014a, 2014b), signal-perception coevolution may shape relative pitch processing skills in much the same way it appears to hone processing related to absolute pitch.

Auditory perception skills can vary with absolute pitch itself in black-capped chickadees. For example, Wong and Gall (2015) demonstrated that auditory sensitivity is at its highest in the range of 2-4 kHz, matching the range of chickadee vocalizations. While this is a broad range that covers all frequencies used in this study, the experiment only employed seven different frequencies between 500-8000 Hz. Especially considering that only one of the seven frequencies (3000 Hz) used by Wong and Gall (2015) fell within the frequency range of chickadee songs (2770-3630 Hz), it may be useful to examine sensitivity more extensively within the species' natural vocal range. If sensitivity differences exist within that range, they could conceivably contribute, along with variation in relative pitch processing, to the discriminative differences observed in this study.

While discrimination of normal songs from those missing the inter-note interval was best at an intermediate frequency (3250 Hz), discrimination of normal songs from those missing the *glissando* within the *fee* note was best at the highest frequency used (3350 Hz). The reason for this difference is not clear, but might relate to the birds employing different strategies for each of the discriminations. While songbirds generally appear to be better at absolute pitch processing than relative pitch processing and rely on it to a greater degree (e.g., Page et al., 1989), there is evidence that they can employ both strategies concurrently. For example, MacDougall-Shackleton and Hulse (1996), in examining the relative importance of these two strategies in European starlings, trained birds on an operant task that could be accomplished using either absolute or relative pitch processing and then exposed them to probe and transfer stimuli designed to clarify which strategy was being employed. Their results indicated that, while absolute pitch processing did take precedence, the birds extracted – and applied during subsequent probe and transfer sessions – both absolute and relative pitch information from the stimuli. If black-capped chickadees use absolute pitch cues to recognize or assess one of the pitch ratios and relative pitch cues to assess the other – or, perhaps more likely, use a different balance of absolute and relative pitch processing for each ratio – it follows that optimal performance may fall within different frequency ranges. In partial support of this, there was no absolute frequency at which discrimination of the songs with both alterations (FFFI) was clearly best, as would be expected if both strategies were employed for this particular discrimination. As suggested above, further work is required in order to understand how both absolute and relative pitch processing function at varying frequencies, as well as how they may be combined in song perception.

Overall, our understanding of how songbirds employ absolute and relative pitch processing, separately and together, remains poor. While songbirds such as starlings appear to rely on absolute pitch processing over relative pitch processing, it is not clear how true that is in species for which relative pitch ratios within songs contain important information (e.g., chickadees, veeries, and white-throated sparrows). Especially considering the evidence that song structure can influence perceptual abilities over evolutionary time (Velez et al., 2014), it may be that the balance between strategies, in terms of skills and employment, differs in such species. Future studies may disentangle the strategies in species such as black-capped chickadees using tasks like those employed by MacDougall-Shackleton and Hulse (1996) in order to establish which type of pitch information is being used.

The previous chapter examined neural and behavioural responses to the same normal and altered song stimuli using a different experimental setup. I quantified immediate early gene (*zenk* protein) activity in two brain areas, caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM), which respond preferentially to conspecific vocalizations. I did not find differences in either area in response to the different stimuli, suggesting that discriminations more subtle than conspecific versus heterospecific (e.g., pitch ratio rules) occur in other parts of the avian auditory pathway. For example, there is some evidence, including operant work, suggesting that the magnocellular nucleus of the neostriatum (lMAN) may be involved in discriminations among conspecific songs (e.g., Scharff et al., 1998).

In conclusion, the current study demonstrated that male and female black-capped chickadees can discriminate songs on the basis of the inter-note pitch interval with relative ease, but had considerable difficulty doing so based on the presence or absence of the *glissando* within the *fee* note. In addition, I found that females were overall better at learning the operant discrimination task, and that the discrimination task was learned better at some absolute frequencies than others. Future studies may clarify this possibility by examining the full range of song production (2770-3630 Hz; Christie et al., 2004b). Most importantly, further exploring the importance of the two pitch ratios to male and female chickadees will shed light on our unclear picture of which pitch ratios are important not just for conspecific recognition but also transmitting information related to individual identity and quality. This would best be accomplished via studies that include both males and females, use biologically relevant response measures (e.g., a preference task in females), and, when possible, combine field and lab methodologies.

CHAPTER 4: REPERTOIRE COMPOSITION AND SINGING BEHAVIOUR IN TWO EASTERN POPULATIONS OF HERMIT THRUSH (*CATHARUS GUTTATUS*)

4.1 INTRODUCTION

Birdsong plays a critical role in reproductive behaviour among songbirds. Sung primarily by males, it serves not only to defend territories against rival males but also to attract females for mating (Catchpole and Slater, 2008). There is an incredible amount of variety across different songbird species with respect to what they sing and how they do so. Songbirds vary with respect to the structural complexity of their songs, how many different forms of song they sing, and many other aspects of singing behaviour (Catchpole and Slater, 2008). Thus, in developing an understanding of birdsong that is comprehensive and reflects such variety, there is great value in studying a wide array of songbird species.

The hermit thrush (*Catharus guttatus*) is a migratory forest songbird that breeds in mixed woods throughout Canada and the western and northeastern United States and winters in moist forests across the southern United States, Mexico and Guatemala (Jones and Donovan 1996). Its song, used throughout the breeding season, consists of a single whistled introductory note followed by a more variable series of flourishes (Jones and Donovan 1996; Figure 4.1). We know little about the organization of their song repertoires, the structure of individual song types, and how males use their repertoires to communicate during the breeding season. This research seeks to describe these features of hermit thrush song, and ultimately to understand how hermit thrush males use their song to maintain territories and attract mates. Providing a basic description of hermit thrush singing behaviour will enable researchers to use this species as a subject for studies addressing more complex topics in birdsong, such as the role of song variation in speciation (Slabbekoorn and Smith, 2002a) and the use of song as an aggressive signal (Searcy and Beecher, 2009).

Previous research on hermit thrush song suggests that male repertoires consist of 6-12 song types that are delivered with immediate variety (Rivers and Kroodsma 2000), meaning no one song type is repeated consecutively. Past studies also suggest that males

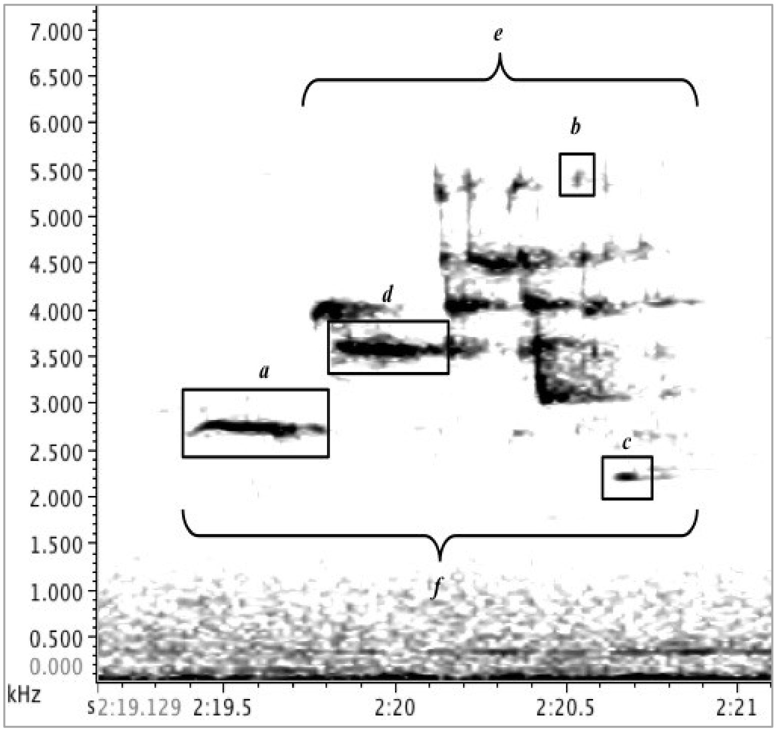

Figure 4.1 Example of a single hermit thrush song type. Basic structural characteristics measured for each song type are indicated: (a) frequency and duration of introductory note, (b) maximum and (c) minimum frequencies, (d) frequency of the post-introductory note of maximum power, and durations of (e) the post-introductory portion and (f) the entire song type.

sing with a degree of predictability, preferring some song type sequences over others but not delivering their song types in a singular, fixed order (Dobson and Lemon 1979; Rivers and Kroodsma 2000). Limited investigation suggests that neighbouring males do not share song types and therefore possess unique song type repertoires (Rivers and Kroodsma 2000).

Despite the hermit thrush's extensive breeding range, which extends across the continent of North America, we know little about how hermit thrush singing behaviour varies within and between the 10-13 subspecies reported (Aldrich 1968, Phillips 1991). Geographic variation in song can be an important marker of different subspecies and play a role in speciation (Slabbekoorn and Smith 2002, Podos and Warren 2007). Rivers and Kroodsma (2000) recorded hermit thrush males from geographically separate populations representing two subspecies: *Catharus guttatus faxoni* in New England and *Catharus guttatus auduboni* in Arizona. They found significant differences between these populations with respect to temporal and frequency-related aspects of song structure

(Rivers and Kroodsma 2000). Variation in singing behaviour occurs not just between subspecies but also within them, as is the case with white-crowned sparrow (*Zonotrichia* leucophrys) song dialects (Baker and Thompson, 1985). Significant differences in rates of song sharing among eastern populations of song sparrows represent another example of variation of singing behaviour within a subspecies (Hughes et al. 1998, Foote and Barber 2007). Among thrushes, song structure differs between geographically separate populations belonging to the same subspecies of Swainson's thrush (*Catharus ustulatus*) (Ruegg and Smith, 2006).

The main objective of the current study was thoroughly to describe repertoire composition, song type structure, and singing behaviour in geographically separate populations of hermit thrush and, in doing so, provide further insight into questions raised by previous work related to geographic variation and song sharing. Birds were recorded from two populations (in and around Halifax, Nova Scotia, Canada and in southeastern Maine), both belonging to the same *C. g. faxoni* subspecies, which covers most of the species' eastern breeding range and much of its breeding range across Canada (Aldrich 1968, Phillips 1991). This allowed me to compare geographic variation across the range of the species without introducing the potentially confounding variable of subspecies. Recordings were analyzed with respect to song type structure, repertoire size, and song syntax. I characterized structurally distinct song type categories, detected some differences in song type structure between the geographically separate populations, and, interestingly, found evidence of within-season changes in singing behaviour.

4.2 METHODS

4.2.1 Data Collection

Spontaneously singing hermit thrush males were recorded on their territories during the 2010 breeding season (17 April – 18 June) using a Marantz PMD 670 solid-state digital recorder (PCM,44.1kHz and 16 bit) with a Sennheiser ME66 directional microphone. Individuals were recorded in the Halifax Regional Municipality, NS, Canada (HFX) and in Hancock County, ME, USA (ME). I recorded 11 individuals in HFX and seven individuals in ME. Birds recorded in Halifax were located in various parks throughout the municipality; four of the 11 males were located within relatively close proximity (100-500 m) to each other, while the others were more distant from each other. In Maine, five of the seven birds recorded were neighbours located along 600 m of a single rural road, while the other two were located approximately 10 km away from the others and approximately 100 m from each other. Because song type sharing between individuals is minimal or non-existent (Rivers and Kroodsma, 2000; Jones, 2005), individuals could be identified on the basis of their song types. All recordings were made during the dawn and dusk choruses.

Previous work indicates that individuals sing with immediate variety and present all of their song types within about 60 songs (Rivers and Kroodsma, 2000; Roach and Knapton, unpublished manuscript). Therefore, in order to capture the full repertoire of each individual, only recordings containing song bouts of at least 100 consecutive songs from the focal individual were selected for analysis. All spectrographic analyses of recordings were completed using RavenPro 1.3 audio analysis software for Mac.

4.2.2 Repertoire Composition

As with previous research on hermit thrush singing behaviour (e.g., Rivers and Kroodsma, 2000), songs (approximately 1.5-second units of singing, temporally separated from other units within the bout) within each bout were categorized into song types; a song type was defined as a unit of song (approximately 1.5 seconds in length) within a bout that is bioacoustically distinct from other song units presented during the bout (Catchpole and Slater, 2008). An individual’s song type repertoire was defined as the collection of the distinct song types sung by that individual.

Spectrograms were generated in RavenPro (Hann window size 1600 samples); brightness and contrast were adjusted to provide a clear visualization of the vocalizations while minimizing the visibility of any background noises. A researcher experienced with reading spectrograms and with hermit thrush song classified recorded songs into song types primarily via visual inspection of spectrograms generated in RavenPro: to aid in this, spectrograms of individual songs were overlaid using GNU Image Manipulation Program (GIMP) 2.6. Songs were categorized as being the same song type if at least 90%

of their notes matched each other. There was little ambiguity in song type classification, as each bird's various song types were very structurally distinct from each other.

To determine inter-rater reliability, a novice experimenter with minimal experience reading sonograms and with hermit thrush song categorized song types for three individuals that the experienced experimenter had already analyzed. There was a 100% match between the repertoire sizes calculated by the experimenters.

4.2.3 Analysis of Song Structure

In order to compare song structure within and between populations, several structural characteristics were measured for all song types observed (n = 96 song types from 11 individuals for HFX; n = 65 song types from 7 individuals for ME). The following parameters related to frequency and duration were measured for each song type: frequency of the introductory note, maximum and minimum frequencies, frequency of the post-introductory note of maximum power, and durations of the introductory note, the post-introductory portion and the entire song type (Figure 4.1). These parameters were chosen in order to provide a broad characterization of hermit thrush song types and to mirror those used by Rivers and Kroodsma (2000).

Measurements were made in RavenPro using selection tables and Hann window sizes of 1600 samples for frequency-related parameters and 256 samples for temporal parameters; these settings were chosen in order to maximize the resolution for each particular measurement. For each song type, structural characteristics were measured for four replicates within a bout and then averaged; in cases of rare song types that occurred fewer than four times (5 of 161 total song types, or 3.1%), only two or three replicates were available for measurement.

4.2.4 Singing Behaviour

Singing rate for each song recording was calculated by dividing the total number of songs during song bouts by the total amount of time over which they were delivered. During continuous singing, the amount of time between song types was very consistent (2.571 ± 0.6094 s; calculated based on all 161 song types recorded). Thus, in order to calculate singing rates that were representative of how quickly individuals actually sang, long pauses between song types (greater than 10 seconds) were not factored into this calculation. Individuals sang at a similar rate during bouts in both populations (Table 4.1).

To characterize the order of song type delivery within song bouts, I generated transition matrices and visualized them using transition diagrams (e.g., Ferreira et al., 2006). Matrices were based on transition tables that enumerate how often each possible song type-to-song type transition occurs within a sequence.

In order to quantify singing patterns, I adapted and calculated the stereotypy score formulated by Scharff and Nottebohm (1991) for quantifying note sequences in zebra finch song. The stereotypy score is calculated as the average of two separate measures: song consistency, which measures how often the most common transitions occur and is equivalent to the predictability score calculated by Rivers and Kroodsma (2000), and song linearity, which is a measure of how many of the possible transitions between song types actually occur. In each case, a higher value indicates a greater amount of predictability within the song type sequence, such that a bout containing the same sequence of song types presented repeatedly without variation would have a stereotypy score of 1 (the stereotypy score for random song type ordering would be close to zero and depend on repertoire size). Because the total number of song types in a bout could affect the stereotypy score by influencing the number of different transitions that occur, these scores were calculated for a 100-song segment of each bout.

Stereotypy score is calculated as the average of the song consistency and song linearity scores, which are calculated as follows:

$$Song\ consistency = \frac{\sum typical\ transitions}{\#\ of\ transitions}$$

$$Song\ linearity = \frac{\#\ song\ types}{\#\ unique\ transitions\ observed}$$

4.2.5 Statistical Analysis

Statistical analyses were completed using Prism 5; an alpha level of 0.05 was used for determining statistical significance. Comparisons between populations were conducted using unpaired t-tests and analyses involving multiple independent variables were conducted using analyses of variance (ANOVA). Pearson correlations were used to examine the associations between singing behaviour and date during the breeding season.

4.3 RESULTS

4.3.1 Repertoire Composition and Song Structure

Repertoire size, defined as the number of distinct song types presented by an individual, ranged from 7 to 12 song types and did not differ between HFX and ME birds (unpaired t-test: t(16)=0.9129, p=0.3748; Table 4.1). There was no correlation between the number of songs in an individual bout and the repertoire size measured for that individual (Pearson correlation: r=-0.2754, p=0.2686; all recordings pooled), suggesting that the calculated repertoire size did not depend on the number of songs analyzed. The number of songs presented from the beginning of an individual's bout until all of its song types were observed (17.44 ± 9.00 songs across all recordings) was far less than the minimum song bout length (100 songs). Together, these data give me confidence that complete song type repertoires were captured. Figure 4.2 shows the song types comprising one individual's repertoire. A comparison of song types among repertoires (both within and between populations) revealed no overlap between individual repertoires. That is, no song type sharing was observed, as no one distinct song type appeared in the repertoire of more than one bird.

Table 4.1 Geographical comparison of characteristics (mean ± SD) related to repertoire organization and singing behaviour. "Gap" refers to the gap in introductory note frequencies between high and low song types within each repertoire; "% low Songs" refers to the percentage of the songs in each bout that were low song types. Statistics show the results of unpaired t-tests.

Measure	**HFX (n=11)**	**ME (n=7)**	**Significance**
Repertoire			
Size (song types)	8.73 ± 1.35	9.29 ± 1.11	p=0.3748, NS
Gap (Hz)	1042 ± 338.2	832 ± 302.3	p=0.2013, NS
Singing behaviour			
Song rate (songs/min)	15.07 ± 2.21	14.73 ± 1.47	P=0.4703, NS
Stereotypy	0.369 ± 0.052	0.372 ± 0.071	p=0.9001, NS
Consistency	0.477 ± 0.066	0.506 ± 0.099	p=0.4632, NS
Linearity	0.260 ± 0.043	0.239 ± 0.061	p=0.3923, NS
% Low songs	64.13 ± 3.010	68.45 ± 5.106	p=0.4468, NS

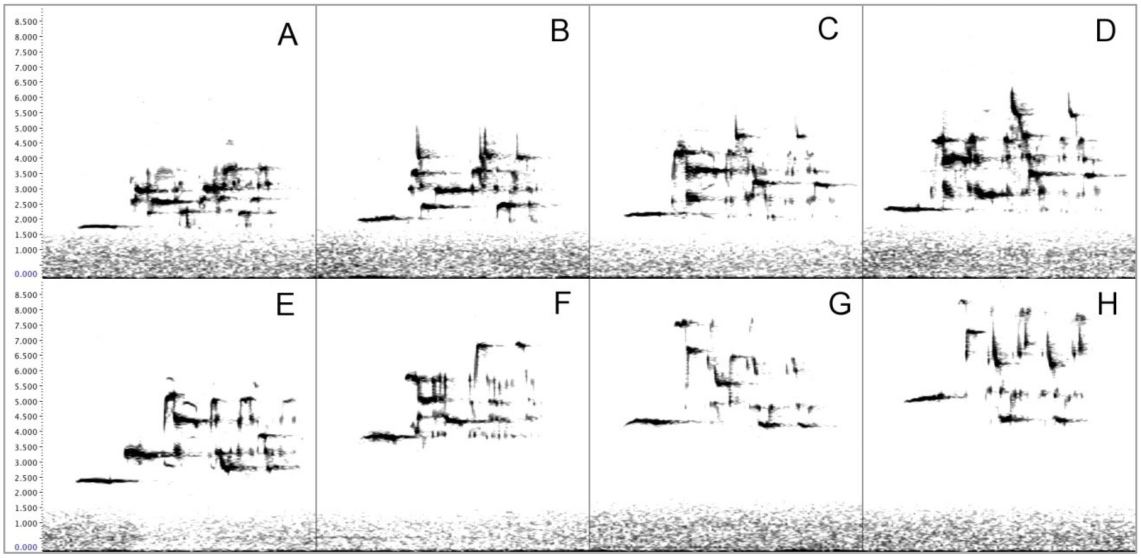

Figure 4.2 Example of an individual's song type repertoire. Each panel represents a unique song type and song types are arranged in ascending order based on introductory note frequency.

Across individuals, song repertoires followed similar organization: each contained multiple song types clustered around two introductory note frequencies, 2000 Hz (Figure 4.2, A-E) and 4000 Hz (Figure 4.2, F-H). Overall, introductory note frequencies among all song types observed ranged from 1617-5062 Hz. However, in each population there was a distinct gap in the distribution of introductory note frequencies (ranging between about 3000-3400), such that introductory note frequencies were not normally distributed throughout the overall range of introductory note frequencies (D'Agostino and Pearson omnibus normality test: K2=157.9, p<0.0001) (Figure 4.3). Across individual repertoires, the size of the gap varied greatly but did not differ between populations (unpaired t-test: (t(16)=1.3333, p=0.2013; Table 4.1). Each song type could thus be classified as a high or low song type based on whether its introductory note frequency was above or below the observed gap (Figure 4.3).

Low and high song types differed structurally: low song types contained more spectral energy spread over a smaller frequency range compared to high song types. To compare low and high types, I calculated each song type's frequency range as the difference between its minimum and maximum frequency (Figure 4.2 C,D). A two-way song type x location ANOVA revealed a main effect of song type (F(1,157) = 21.88, p<0.0001) but not location (F(1,157) = 0.25, p=0.61), with no interaction (F(1,157) =189,

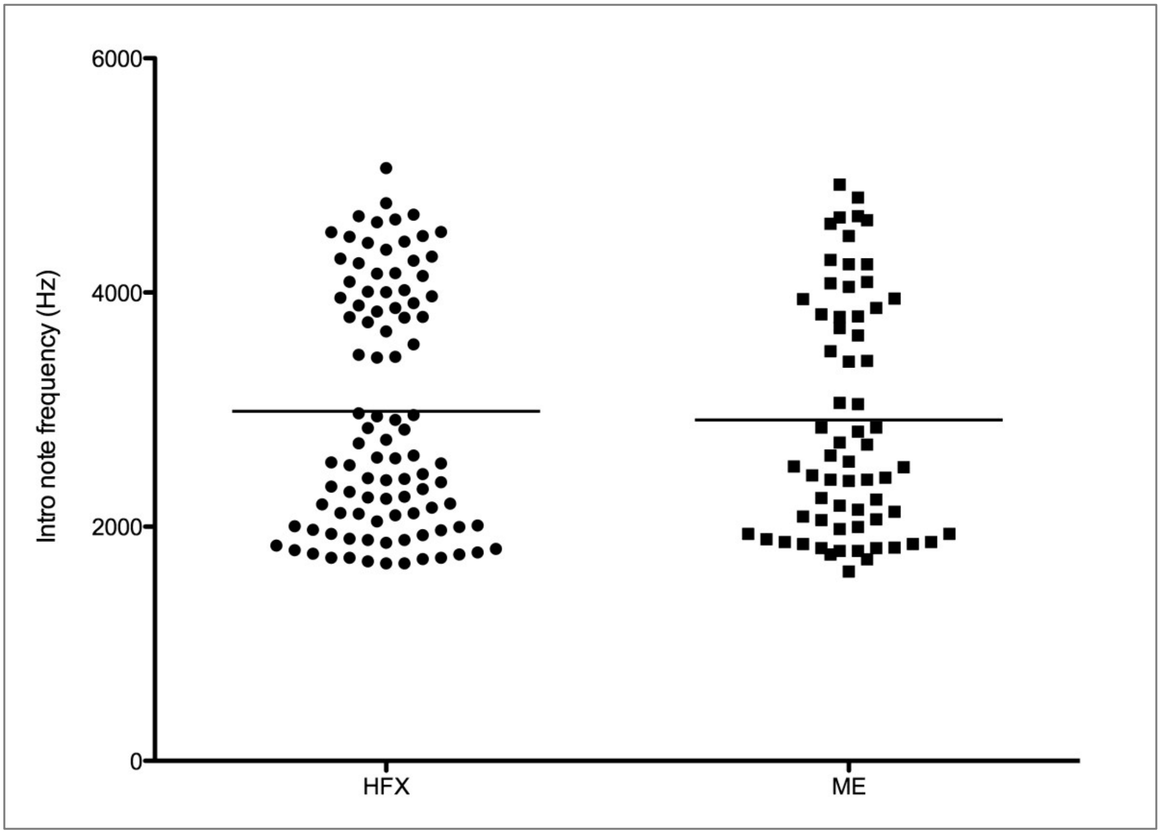

Figure 4.3 Distribution of introductory note frequencies of song types in the HFX (n=96) and ME (n=65) populations. Horizontal positioning of points has no significance; horizontal line represents mean.

p=0.17). Frequency range was greater in high song types compared to low song types for both HFX (high: 2965 ± 574 Hz, low: 3560 ± 694 Hz) and ME (low: 3050 ± 519 Hz, high: 3375 ± 592 Hz).

In addition to the difference in frequency range, visual inspection of spectrograms suggested there was less overall spectral energy in high song types compared to low song types. To address this, I compared high and low song types with regard to total amount of energy in the post-introductory portion of the song using the Average Power measurement in Raven, which calculates the density of spectral energy in a sample. The power within the post-introductory portion was normalized by subtracting the power of the introductory note, thus producing a value reflecting the energy levels in the post-introductory portion relative to the introductory note. This was calculated for five recordings from each population that were particularly devoid of background noise that could affect the energy measurements. A two-way song type x location ANOVA revealed a main effect of song type (F(1,85) = 52.72, p<0.0001) and of location (F(1,85) = 5.20, p=0.015) and no interaction (F(1,85) = 0.89, p=0.35), with song type category accounting for 53.5% of the total variance. Post-introductory energy (relative to the introductory note) of low song types was higher than that of high song types in both HFX (low: 9.842 ± 4.027 dB, high: -0.3647 ± 3.755 dB) and ME (low: 6.724 ± 4.250 dB, high: -1.766 ± 4.301 dB).

Geographical comparison of basic song type characteristics between populations is summarized in Table 4.2. Song types from the two populations did not differ significantly with respect to any of the frequency-related parameters. The two populations did, however, differ significantly with respect to temporal parameters: the duration of the introductory note, post-introductory notes, and entire song-type, was longer for HX than ME birds (see Table 4.2).

Table 4.2 Geographical comparison of basic song type characteristics (mean ± SD) related to frequency (introductory note, post-introductory note, the difference between them (Change), maximum, minimum, range) and duration (introductory note, post-introductory portion of song type, entire song type). Statistics show the results of unpaired t-tests.

Measure	HFX (n=96)	ME (n=65)	Significance
Frequency (Hz)			
Intro	2986 ± 1033	2911 ± 1008	p=0.6520, NS
Post-intro	4037 ± 1168	3854 ± 989	p=0.3038, NS
Change	1094 ± 648	953 ± 464	p=0.1060, NS
Max	5869 ± 1370	5790 ± 1245	P=0.7079, NS
Min	2663 ± 940	2619 ± 1004	p=0.7798, NS
Time (s)			
Intro	0.3040 ± 0.0393	0.2720 ± 0.4103	p<0.0001, ***, R^2 = 0.14
Post-intro	1.112 ± 0.1703	1.050 ± 0.1893	p=0.0334, *
Whole song	1.416 ± 0.1816	1.322 ± 0.1958	p=0.0023, **

4.3.2 Singing Behaviour

All individuals sang with immediate variety: there were zero instances of the same song type being used twice consecutively. Within each song bout, any given song type was followed by one of only a handful of the other song types from the repertoire, such that some transitions occurred much more frequently than others while many transitions did not occur at all (Figures 4.4 and 4.5).

The order of individual song type delivery was neither completely random nor fully predictable: the stereotypy score (and its component scores) varied greatly across individuals (ranging from 0.269 to 0.508 across all individuals), but there was no geographical difference evident for any of the three measures (Table 4.1). I examined whether this measure of song type delivery order varied depending on time of day or across the breeding season. There was no difference with respect to these measures between song bouts recorded at dawn versus dusk (unpaired t-test for stereotypy score: t(16)=1.15, p=0.2813). There was also no relationship between recording date and stereotypy score among song bouts recorded in Halifax (Pearson correlation: r=-0.526; p=0.0961), although song linearity scores did decline significantly over the season (Pearson correlation: r=-0.6792, p=0.0215) (Figure 4.6).

Given the division among song types within each repertoire, I also quantified the use of high songs versus low songs within song bouts by calculating the percentage of the total songs presented in each individual's song bout that were low song types (defined as song types with introductory frequencies below the above-described gap). As with stereotypy

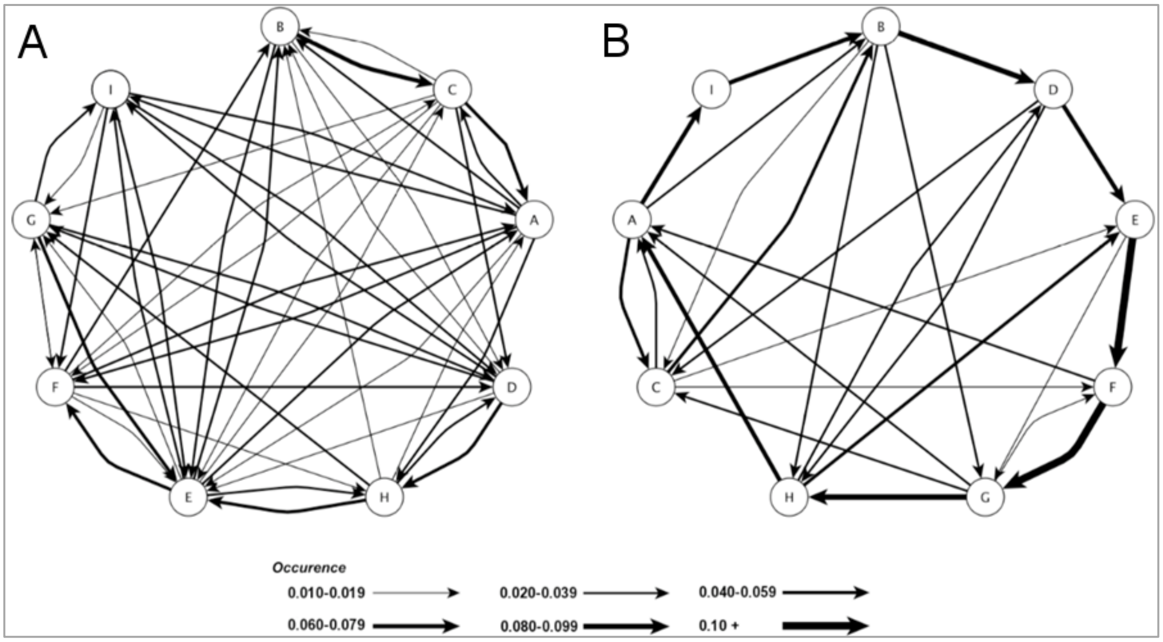

Figure 4.4 Transition diagrams for the individuals with the lowest (A) and highest (B) observed stereotypy scores (0.27 and 0.51, respectively), showing the relative occurrence of all possible song type-to-song type transitions in each song bout. Letters represent song types, and arrow size represents occurrence (calculated as proportion of the total number of transitions); any bending of arrows is done for visual clarity and has no further significance.

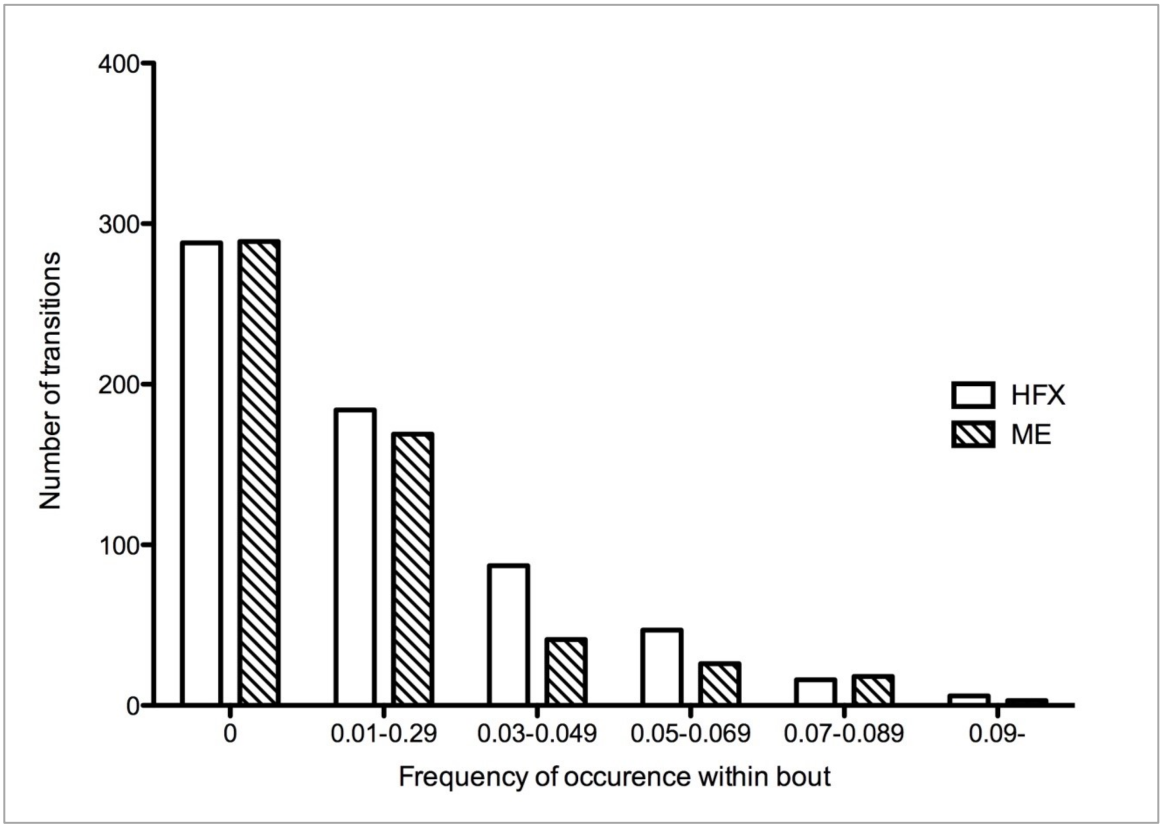

Figure 4.5 Histogram showing the relative occurrence within bouts of all possible song type-to-song type transitions; includes all individuals.

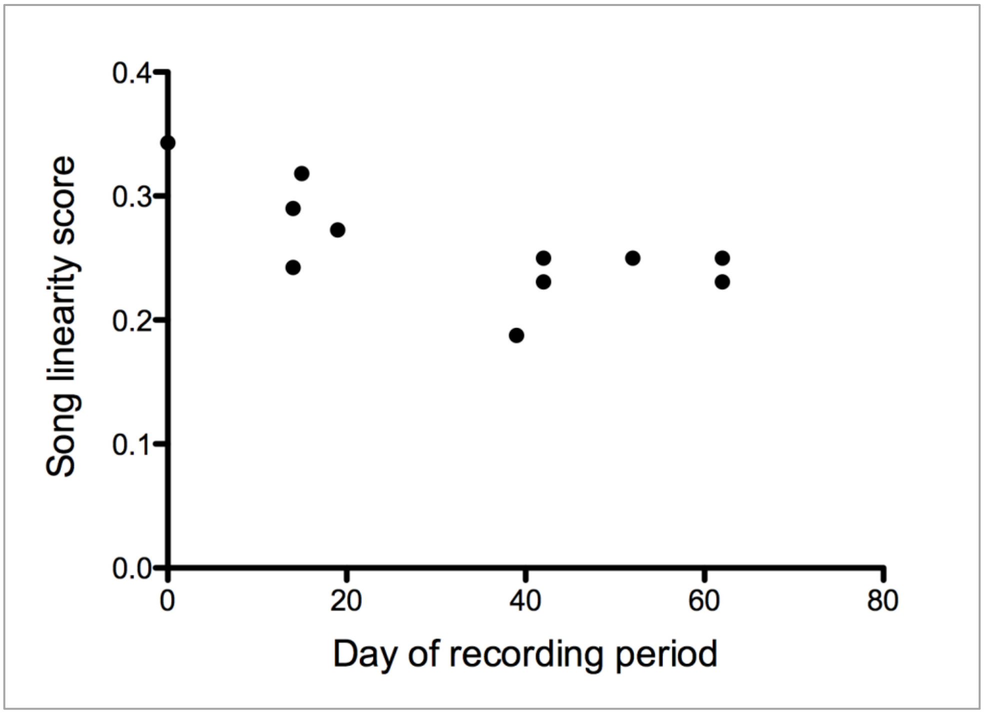

Figure 4.6 Scatterplot showing song linearity scores over the recording period (with day 0 being April 26) for Halifax recordings (n = 11).

score, the percentage of low song types in each song bout varied greatly (43-89% across all individuals) but did not differ between populations (Table 4.1). Song bouts recorded in near dawn contained a greater proportion of low song types compared to those recorded at dusk (t(16)=2.47, p=0.025). In addition, the proportion of low song types within Halifax bouts declined significantly over the course of the breeding season (Pearson correlation: r=-0.6106, p=0.046) (Figure 4.7). That is, song bouts recorded earlier in the breeding season contained a greater proportion of low song types compared to those recorded later in the season.

All males alternated between high and low song types during bouts, with single high song types typically being followed by one to a few low song types before delivery of another high song type (Figure 4.8). Consecutive high song types occurred very rarely, with only six instances recorded across all song bouts (totaling 1800 transitions). To quantify this cycling pattern, I calculated new consistency scores using transition matrices representing just two states (high and low song types) rather than all distinct song types. Using just these two transition states allows for a measure of the predictability with which individuals deliver high versus low song types. These

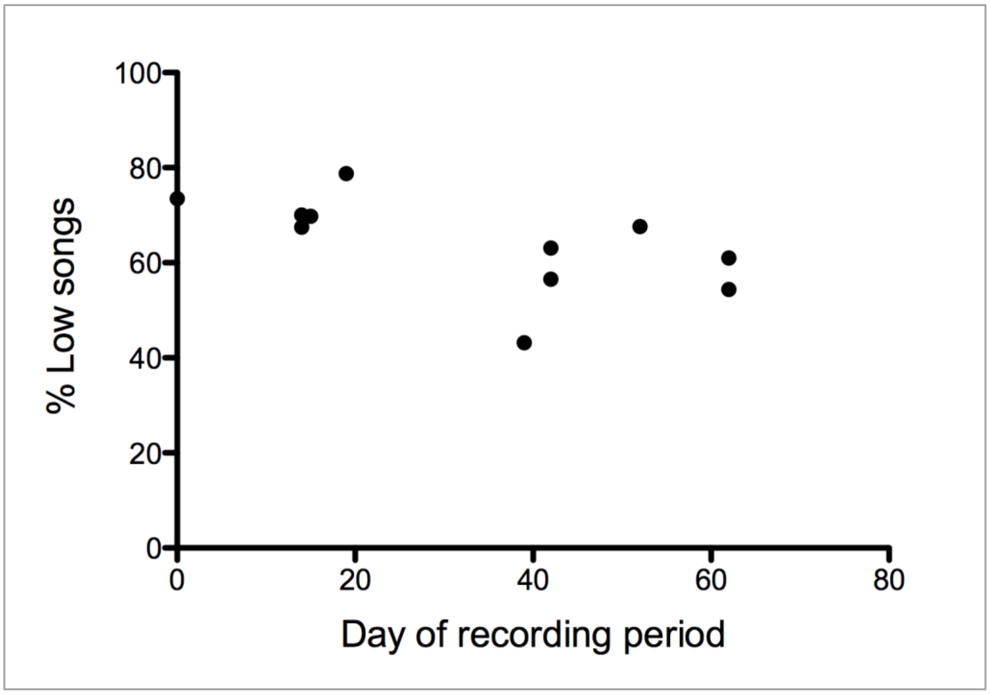

Figure 4.7 Scatterplot showing the percentage of low songs (the number of songs within a bout that were low song types) over the recording period (with day 0 being April 25) for Halifax recordings (n = 11).

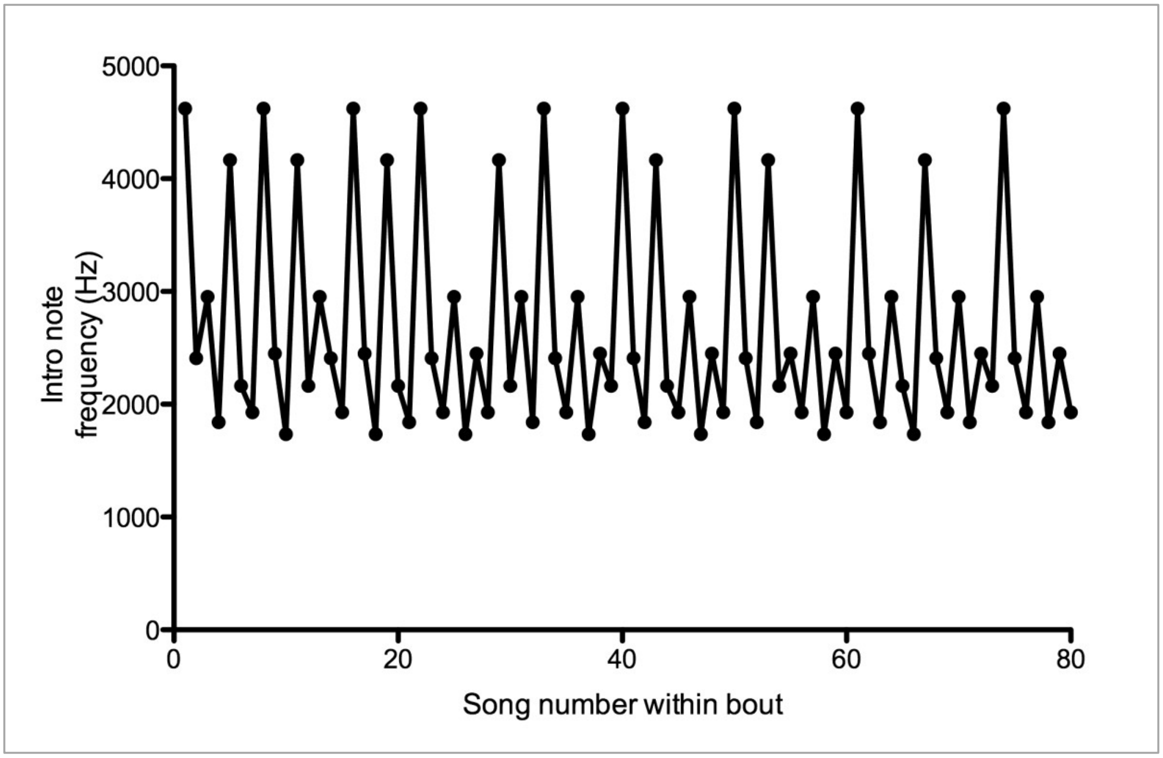

Figure 4.8 The sequence of song types, represented by their introductory note frequencies, presented during a portion of a song bout by a representative individual.

consistency scores were higher than those previously calculated, ranging from 0.67-0.93, and did not differ between populations (Table 4.1).

4.4 DISCUSSION

This paper provides a basic description of repertoire composition, song type structure, and song type delivery in singing bouts recorded from spontaneously singing hermit thrush males in two geographically distinct populations. The song types in each individual's repertoire could be sorted into high and low categories based on the frequency of their introductory notes, and these categories differ structurally from each other. While some differences between populations were detected in terms of song structure, the syntax of song type presentation appeared the same in each case: Males sang with immediate variety and presented song types in an order that was neither random nor fully predictable.

4.4.1 Song Type Repertoires and Sharing

The repertoire sizes observed in this study, ranging from 7-12 song types, are similar to those described in previous research on hermit thrush song type repertoires: Borror (1964) reported up to 13 song types per individual, and Rivers and Kroodsma (2000) found that repertoire size ranged from 6 to 12. As with Rivers and Kroodsma (2000), I found no evidence of song type sharing within or between populations – either in HFX, where the recorded males tended to be non-neighbours, or in ME, where most of the males were neighbours to each other and located along a single stretch of road.

Although the lack of song sharing evidence here and in previous work suggests that it occurs rarely, if at all, further work is needed to determine whether individuals share smaller song elements (e.g., notes or syllables). This is especially important to understanding how males use their songs during the breeding season, as song type sharing may be important to male-male interactions in that it allows for song matching between males. Many studies in other songbirds (e.g., great tits, Krebs et al. 1981; western song sparrows, Stoddard et al. 1992) suggest that song type matching acts as an aggressive signal between males, although the results across all songbirds are not conclusive (see Searcy and Beecher 2009). Even in species where males do not share exact song types, an individual may match a conspecific using song types with shared song elements (e.g., song sparrows, Anderson et al. 2005) or types of similar frequency (frequency matching; e.g., Harris's sparrows, Shackleton et al. 1991). Thus, even in the absence of song type sharing, it is possible that hermit thrush males engage in a form of song matching, either via shared song elements or by using song types of similar frequency.

4.4.2 High and Low Song Type Categories

I found that the song types within each male's repertoire could be divided into high and low song type categories based on their introductory note frequency, and that these song types differ structurally. It is possible that high and low song type categories serve divergent functional roles related to territorial defense and mate attraction, especially given that high frequency sounds degrade more quickly and are therefore not as useful for long-range communication (Wiley and Richards 1978). Such splits have been suggested in other songbirds. For example, great tit (*Parus major*) males use their lowest frequency songs when females are at their fertility peak (Halfwerk et al. 2011) and sing higher frequency songs when the density of male conspecifics is greater (Hamao et al. 2011). In addition, song quality at low frequencies is an important indicator of male quality in black-capped chickadees (Christie et al. 2004). Lastly, *Acrocephalus* warblers use complex song types for courtship and more simple song types for territorial male-male interactions (Catchpole 2000).

Considering these results, it is tempting to hypothesize that hermit thrush song type categories represent a similar functional split, with simple high frequency songs directed at male rivals that are threateningly close to the singing male's territory and more complex low frequency songs directed at potential mates (and possibly more distant neighbours) that may be located at greater distances. One difficulty with this interpretation is that singing males cycle through high and low song types in one song bout, rather than singing one or the other type exclusively. There was, however, considerable variation in the relative proportion of these types among recorded bouts, with the proportion of low song types within singing bouts decreasing over the breeding season.

Specifically, the proportion of low song types within bouts declined over the course of the breeding season. It is difficult to draw firm conclusions from this finding because it is correlational and represents different individuals recorded throughout the seasons rather than recordings made of the same individuals at different time points within the season. It may be that individuals do in fact sing more low songs earlier in the season, possibly related to the functional divergence of high and low song types discussed above. Alternatively, the observed differences may be due to different individuals being vocally active at different points in the breeding season. Bent (1949) reported two peaks in hermit thrush singing activity during the breeding season; this second peak may be associated with a second brood attempt by some individuals (Jones and Donovan 1996). Future work using song playback and multiple recordings of individuals at different stages in the breeding season will address these possibilities, and will help clarify whether high and low song types serve divergent functions.

4.4.3 Song Type Delivery

Hermit thrush males sang with immediate variety in their song types, cycling between high song types and clusters of one to a few low song types (in concurrence with other hermit thrush work (Rivers and Kroodsma 2000; Kroodsma 2005)), and displayed distinct preferences in terms of song type-to-song type transitions. Based on song consistency scores, song type sequencing within song bouts is about 50% predictable; that is, one would be successful about half the time in predicting the next song type in a sequence based on which transitions are most common. If the sequence of song types within bouts were random, I would expect predictability scores ranging between 9-17% (for repertoire sizes ranging from 7-12 song types, assuming immediate variety). Consistency scores in this study were comparable to the similarly calculated predictability values found in New England birds by Rivers and Kroodsma (2000). They were also consistent with Dobson and Lemon (1977), who used Markov analysis to examine song type sequences in a few hermit thrush males. They found that hermit thrush song sequences contained elements of both first- and second-order Markov sequences, suggesting that the song type used at any point depends partly but not completely on the one preceding it.

The transition preferences seen here are similar to those seen in wood thrushes (*Hylocichla mustelina*). Individuals have repertoires of two to eight B phrases (the learned middle component of wood thrush songs), a smaller but comparable number of song type variants to hermit thrush song type repertoires. As with hermit thrush song types, wood thrush males show strong preferences for some variant-to-variant transitions while not producing other transitions at all (Whitney 1985).

The occurrence of preferred transitions in hermit thrush song appears to be a consequence of the overall syntax of its singing, as males repeat patterns containing one high frequency song type followed by one to several low frequency song types. I have shown that males are highly predictable with regard to their switching between high and low song types, although, as with song type-to-song type transitions, there is considerable variation across individuals. Future research will seek gain a fuller understanding of song syntax and its relevance to breeding behaviour, likely also providing an explanation for the variation observed here.

4.4.4 Population Differences

Rivers and Kroodsma (2000) found differences in hermit thrush song between geographically distant populations that represent different subspecies and called for further work studying possible differences in singing behaviour throughout the species' range. Here I compared hermit thrush song from individuals of two populations that are geographically separate but belong to the same subspecies (*C. g. faxoni*). There were no observed differences between the populations with regard to frequency characteristics of the song types, and these measures were similar to those reported by Rivers and Kroodsma (2000) in their New England population of the same subspecies. In addition, no geographical differences were observed in measures of singing behaviour such as singing rate and stereotypy. However, the populations did differ with regard to temporal characteristics.

Song types of HFX individuals were longer than those of ME individuals, and this difference was most pronounced for the duration of the introductory note. One possible explanation for the temporal differences observed relates to habitat differences between the populations. Geographical differences in song characteristics have been linked to habitat differences in other species (e.g., tanagers; Shy 1983), and previous work suggests that temporal characteristics in particular (more so than frequency characteristics) are strongly linked to habitat within eastern North American songbird species (Wiley 1991). In this study, recording sites were similar between HFX and ME, although territories in HFX tended to be more mixed coniferous and deciduous while ME sites were more fully coniferous. It is therefore possible that differences in vegetation have contributed to differences in temporal song characteristics, possibly via the effects of forest reverberations upon note lengths (Slabbekoorn and Smith, 2002b). Further work is required in order to better understand the effect of habitat upon song structure, both on this scale and across the entire hermit thrush breeding range.

4.4.5 Conclusions

In this paper, I described repertoire composition, song type structure and singing behaviour in two geographical populations representing the same hermit thrush subspecies. Results indicated that, in keeping with findings by Rivers and Kroodsma (2000), individuals have repertoires of 7-12 song types; there was no evidence of song type sharing. The song types that make up individual repertoires can be divided into high and low song types based on introductory note frequency, and males alternate between these types with immediate variety. In addition, some aspects of singing behaviour changed significantly over the course of the breeding season. Lastly, comparison between populations from Maine and Nova Scotia revealed differences in temporal song characteristics.

This paper raised several questions that should be addressed by future work on hermit thrush song. The possible functional significance of the high and low song types described here should be further investigated. Recording of banded individuals at multiple time points throughout the season will address the correlational changes in singing behaviour detected here and also may shed light on the functional significance, if any, of the song type categories described above. In addition, playback-response experiments using recordings with greater proportions of high or low song types might shed light on this question via territorial male response (vocal and physical) to such stimuli.

Another priority for future work, as mentioned above, should be thorough characterization of the degree of sharing of song types (or smaller song elements) between individuals within and between different neighbourhoods. Related to that, the extent of song matching, a possible aggressive signal (Searcy and Beecher 2009), should be examined via recording of natural countersinging between neighbour males as well as playback-and-response to simulate territorial threats by rival males.

CHAPTER 5: GEOGRAPHIC VARIATION IN SONG STRUCTURE IN THE HERMIT THRUSH (*CATHARUS GUTTATUS*)

5.1 INTRODUCTION

While many songbird species produce songs that are consistent in structure throughout most or all of their ranges, the songs of other species can vary substantially depending on location. Because song plays a vitally important role in songbird behaviour, both in territorial defense and in mate attraction (Catchpole and Slater, 2003), geographic song variation can influence the evolutionary trajectory of species. For example, sufficient differences in song between populations can lead to lack of conspecific recognition, contributing to reproductive isolation followed by speciation (e.g, Irwin et al., 2001). Nonetheless, as detailed below, geographic song variation is most often a by-product of evolutionary processes (Podos and Warren, 2007). Regardless of whether song variation is a driving force or a consequence of reproductive isolation and similar events, understanding how and why song varies across a species' range can provide valuable insights into its evolutionary history (Slabbekoorn and Smith, 2002a; Podos and Warren, 2007).

Divergence in song structure can develop across a bird's geographic range through a variety of mechanisms. One of these involves physical adaptations to different ecological conditions that may, in turn, lead to changes in song structure. For example, differences in beak morphology among Darwin's finches emerged as adaptations that optimized feeding in different environments, and these changes in beak shape also produced differences in song characteristics (Podos and Nowicki, 2004; Huber and Podos, 2006). Similarly, selection on body size can affect the physical apparatus of song production (e.g., syrinx size), which in turn influences the sound frequencies that a bird can produce. A number of studies have demonstrated a negative correlation between body size and song frequency (Ryan and Brenowitz, 1985; Bertelli and Tubaro, 2002), with some showing a similar negative association between body size and frequency bandwidth (Mason and Burns, 2015).

Variation in song structure may arise not only as a by-product of other adaptations to the environment but also as a direct adaptation. As the acoustic adaptation hypothesis states, the potential of the habitat itself to attenuate and degrade vocal signals can lead to selection for song structure characteristics that minimize interference and maximize signal fidelity (Wiley and Richards, 1978; Slabbekoorn, 2004). As a result, habitat structure can influence amplitude, temporal and frequency characteristics of song structure (e.g., Wiley, 1991). In terms of song frequency, closed habitats with dense vegetation favour lower frequencies that are less degraded by vegetation, while higher frequencies are more common in open habitats (Wiley and Richards, 1978; Boncoraglio and Saino, 2007; Derryberry, 2009). Habitat structure may even influence the types of vocal elements within songs, as whistles are less degraded by vegetation than trills (Brown and Handford, 2000). In addition to the effects of habitat structure, song structure can also be influenced by other environmental sounds, including other natural sounds (e.g., insects, rainfall; Slabbekoorn, 2004), and noise associated with human activity (Slabbekoorn and den Boer-Visser, 2006).

Song divergence may also arise via cultural transmission of song patterns: song learning in songbirds is not a perfect process, and errors that result from inaccurate copying from tutors may persist within populations, become established, and contribute to geographic variation (Slater, 1989; Lachlan and Servedio, 2004). Sexual selection can also mediate geographic song variation, as differences in female preferences for male song attributes may influence song complexity or other acoustic features across lineages (Podos and Warren, 2007; e.g., Irwin et al., 2001). Geographic variation can also be influenced by differences in factors such as perch height (Lemon et al., 1981) and territory size (Calder, 1990).

It is important to note that these mechanisms of song variation (i.e., physical adaptation, environmental adaptation, and cultural transmission) are not mutually exclusive and may act in concert. For example, song structure differences among Darwin’s finches have been linked both to changes in beak morphology via natural selection (Podos and Nowicki, 2004; Huber and Podos, 2006) and to cultural drift (Grant and Grant, 1996). Likewise, differences in both bill size and habitat contribute to song variation in white-crowned sparrows (*Zonotrichia leucophrys*) (Derryberry, 2009).

In recent years, research on geographic song variation has provided valuable insights into the histories and systematics of *Catharus* thrushes. For example, in the gray-cheeked thrush (*C. minimus*), the structural song differences between *Catharus minimus bicknelli* and other *C. minimus* subspecies was one of the main factors in establishing the former as its own species (Bicknell's thrush, *C. bicknelli*) (Ouellet, 1993). The case for a species split was further bolstered by a lack of species recognition between groups: *C.m. bicknelli* males responded to territorial playback of *C.m. bicknelli* songs but not to those of other gray-cheeked thrush subspecies (Ouellet, 1993).

Researchers have also examined geographic song variation in Swainson's thrush (*C. ustulatus*), in the context of understanding the migratory routes and genetic divergence of its two major geographic groups. Those groups, Coastal and Inland, appear to have been initially segregated from each other by ice sheets emerging during the Early to Mid-Pleistocene era (Ruegg and Smith, 2002; Ruegg et al., 2006a). Although contact between the groups was re-established following dissipation of the ice sheets, Ruegg et al. (2006b) found that populations representing the two groups differ in a number of ways with respect to song structure, and that these differences correlate not with genetic differences but with ecological ones (factors related to rainfall and temperature). In particular, Coastal birds sing longer songs and exhibit lower frequencies; the authors suggest that these song differences may relate to differences in rainfall amounts and vegetation density (Ruegg et al., 2006b).

Relatively little is known about geographic song variation in another, closely related species, the hermit thrush (*C. guttatus*). Males sing songs composed of a single introductory note followed by a complex post-introductory series of notes (Figure 5.1). Individuals have repertoires of 6-12 song types, which they deliver with immediate variety (never repeating the same song type twice) and in a semi-random order (Chapter 4). Existing research suggests that song-type sharing between neighbours occurs either at a very low rate or not at all (Jones, 2005; Chapter 4).

The hermit thrush has a large breeding range that is similar to that of Swainson's thrush, covering most of Canada, as well as much of the western and northeastern United States (Dellinger et al., 2012). There are approximately 12 subspecies, although precise subspecies classification has been a source of debate and would benefit from thorough study using genetic and morphological data (Aldrich, 1968; Phillips, 1991; Dellinger et al., 2012). More broadly, hermit thrush subspecies have been divided into three major geographic groups on the basis of breeding location (Dellinger et al., 2012): Northern, which covers eastern North America and spans across Canada into central British Columbia and central Alaska; Western Lowland, which stretches along the Pacific Coast from Alaska to Baja California; and Western Mountain, which comprises the mountain ranges of the western United States (e.g., Rocky

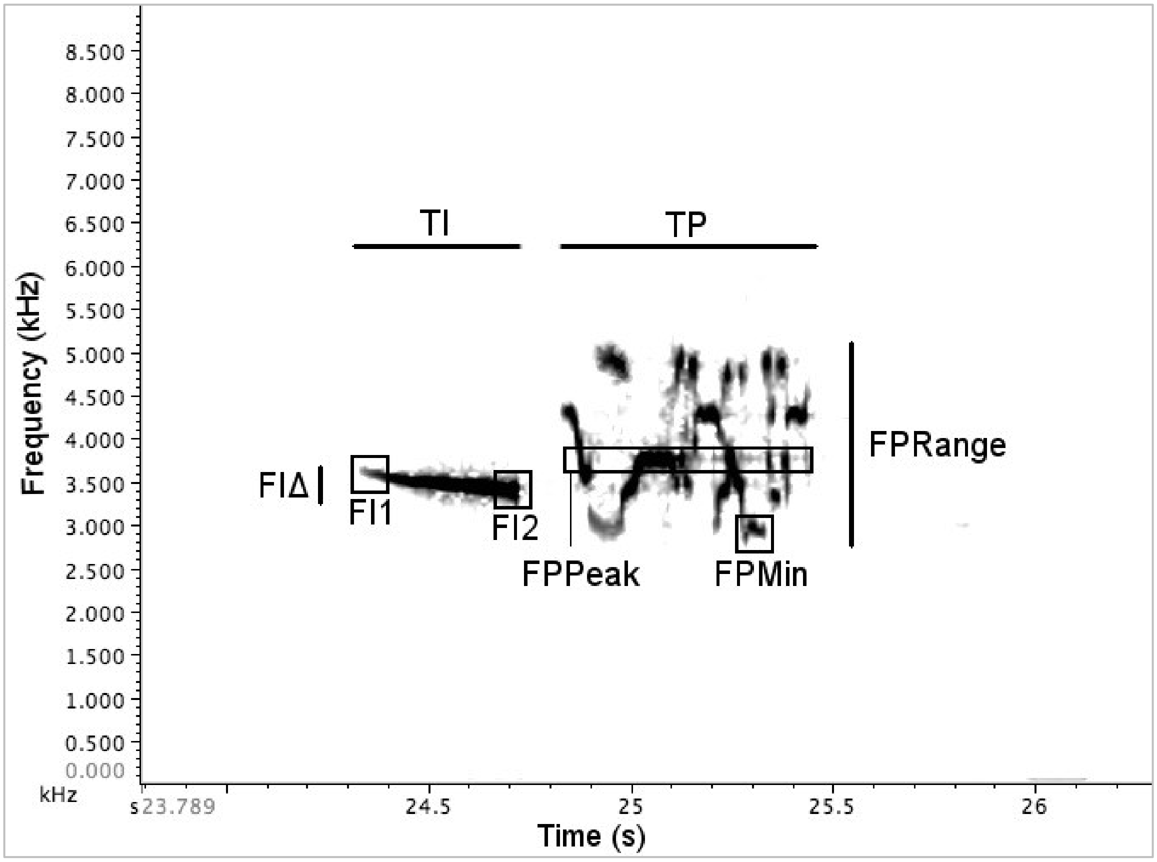

Figure 5.1 Spectrogram of a hermit thrush song type, illustrating the acoustic song variables measured. See Table 5.1 for descriptions of the acoustic variables used.

Mountains, Sierra Nevada). In terms of geographic song differences, Rivers and Kroodsma (2000) found extensive frequency and temporal differences between populations of hermit thrush in Arizona (*C.g. auduboni,* a Western Mountain subspecies) and New England (*C.g. faxoni,* a Northern subspecies). On a smaller geographic scale (Nova Scotia vs. Maine, Roach et al. 2013) found more restricted acoustic differences (related to duration of song elements) between two *C.g. faxoni* populations. However, little is known about overall geographic variation in hermit thrush song structure, and Rivers and Kroodsma (2000) identified this as a priority for future research.

Alvarado et al. (2014) recently used geolocators in combination with genetic and morphological data to examine migratory patterns in hermit thrush. They found a high degree of genetic divergence between western (corresponding to the Western Mountain and Western Lowland groups) and eastern (corresponding to the Northern group) birds, spanning a migratory divide similar in location to that reported in Swainson's thrush (Ruegg and Smith, 2002; Ruegg et al., 2006a) and likely originating from the same glacial separation event one million years ago. The authors suggest that this divide has been maintained, despite secondary contact, in part by the reduced fitness of eastern-western hybrids, a deficit resulting from intermediate migratory routes that reduce survival. Among the other suggested contributors to maintenance of the divide is variation in song structure (Alvarado et al., 2014).

As Alvarado et al. (2014) point out, the high level of divergence observed in their study is reminiscent of other boreal superspecies complexes (groups of newly separated yet highly similar species) that were initially separated by the same ice age-associated fragmentation (Weir and Schluter, 2004). Supporting this idea, barcode analysis of mitochondrial DNA from hermit thrush identified two distinct genetic clusters, representing a level of divergence that surpassed the authors' threshold for a provisional split into two separate species (Kerr et al., 2007). Topp et al. (2013) found a similarly large genetic divergence between western and eastern hermit thrush populations based on analysis of mitochondrial DNA.

The objective of this study was to provide, for the first time, a thorough overview of geographic variation in hermit thrush song structure. Information on how song varies geographically in this species has the potential to inform our currently unclear picture of its subspecies designations and, more significantly, to relate song structure to the genetic and morphological patterns associated with the observed migratory divide (Alvarado et al., 2014). Such integration of information about genetics, morphology and song structure will improve our understanding of the forces shaping the evolution of the species and its taxonomic subgroups. Recordings of songs from throughout the hermit thrush breeding range were collected from a variety of sources and analyzed with respect to song structure. Principal component and discriminant function analyses were used in order to assess the degree of divergence in song structure across hermit thrush major geographic groups.

5.2 METHODS

5.2.1 Recordings

Recordings, each representing a single vocalizing hermit thrush, were collected from the Cornell Lab of Ornithology's MacAulay Library (macaulaylibrary.org), xeno-canto (www.xeno-canto.org), Michigan State University's Avian Vocalization Center (avocet.zoology.msu.edu) and the Ohio State Borror Laboratory of Bioacoustics (blb.osu.edu), as well as number of independent recordists. Recording dates ranged from 1951 to 2015. In cases where the exact GPS coordinates or altitude information was not provided for a given recording, that information was obtained by inputting location information reported by the recordist into Google Earth. Given the lack of evidence of song type sharing in hermit thrush (Jones, 2005; Chapter 4), I was able to use visual inspection of song spectrograms to examine recordings made in close proximity to each other in order to eliminate any duplicate recordings of individuals. The 104 individuals were classified into major geographic group (Northern, Western Lowland, and Western Mountain) and subspecies based on recording location using the detailed geography-based range descriptions of the subspecies making up the groups provided by Dellinger et al. (2012). Three of the individual recordings collected were excluded from the analysis because their geographic group membership was unclear based on the descriptions in Dellinger et al. (2012).

Spectrographic analysis of song structure was conducted using RavenPro 1.4 (Bioacoustics Research Program, 2011), with a Hann window size of 1000 samples. All recordings were digital, although they varied in terms of original recording source and bitrate. For each song type within a recording, I measured eight acoustic variables associated with frequency and duration: frequency at the start (FI1) and end (FI2) of the introductory note, the change in frequency from the start to the end of the introductory note (FIΔ), the minimum (FPMin) and peak energy (FPPeak) frequencies of the post-introductory portion, the bandwidth of the post-introductory portion (difference between maximum and minimum frequency; FPBand), and the durations of the introductory note (TI) and the post-introductory portion (TP) (Table 5.1; Figure 5.1).

In the interest of comparing structure at the level of individuals, and to avoid issues related to pseudoreplication, I calculated each variable's mean across all song types within a recording. Unlike other studies with a similar approach (e.g., Lovell and Lein, 2013), however, the songs within an individual hermit thrush's repertoire can vary greatly in absolute frequency (Chapter 4), such that information could be lost by using only the mean values for each individual. In the interest of capturing such within-individual variation for the six frequency-related variables examined in this study, I calculated not just the mean but also the maximum and minimum for the song types in each recording. With mean values for all eight acoustic variables and maximum and minimum values for the six frequency-related variables, there were a total of 20 acoustic variables used in the subsequent analyses.

Table 5.1 Summary of acoustic variables measured.

Variable	Explanation
FI1	Frequency at start of introductory note
FI2	Frequency at end of introductory note
FIΔ	Absolute change in frequency from start to end of introductory note *Absolute of FI1-FI2*
FPMin	Minimum frequency within post-introductory portion
FPBand	Frequency bandwidth of post-introductory portion *FPMax-FPMin*
FPPeak	Frequency with highest energy within the post-introductory portion
TI	Duration of introductory note
TP	Duration of post-introductory portion of song

5.2.2 Statistical analysis

Because of the uncertainty regarding hermit thrush subspecies designations, initial statistical analyses were conducted on the level of the three major geographic groups

(Northern, Western Mountain, and Western Lowland). Statistical analyses were completed in IBM SPSS Statistics 22 with an α value of 0.05, unless otherwise noted.

To reduce the number of variables and examine how they contributed to song structure variation, I conducted a principal component analysis (PCA). Principal components with eigenvalues greater than 1.00 were used in subsequent analyses. One of the resulting principal components (PCs) lacked within-class normality, as assessed by examining the data's residuals, and could not be made normal by transformation (with log, ln, or square root). As a result, the principal component data did not the meet the multivariate normality assumption of the planned analyses, MANOVA and discriminant function analysis. Based on examination of both the raw data and of plots showing residuals of regression for the principal components, one Western Mountain individual was identified as a multivariate outlier. The principal component analysis was rerun with the remaining 100 individuals; the non-normal distribution was still present.

To address the fact that PC data did not meet the requirements for MANOVA and discriminant function analysis, data were first examined using a classification method that does not require normal distribution, k-nearest neighbor analysis. This approach classifies cases into groups based on the group membership of the most similar other cases. Based on this algorithm's incorrect group membership predictions occurring within two specific sub-groups, the groups were reorganized such that members of the Western Lowland subspecies *C.g. slevini* and high-altitude members of the Northern subspecies *C.g. euborius* were each put into their own group. The resulting five geographic groups (Northern (n = 39 individuals), Western Lowland (n = 17), Western Mountain (n = 32), high-altitude *C.g. euborius* (n = 6), and *C.g. slevini* (n=6)) exhibited within-class normality, thus meeting the requirements for subsequent analyses.

Differences in song structure across these five groups were assessed via MANOVA using the principal components that resulted from the PCA. Between-group differences were examined using Bonferroni-corrected post-hoc comparisons. In order to test whether individuals could be accurately classified on the basis of song structure, I then conducted a discriminant function analysis (DFA) using the principal components described above. Geographic group was used as the grouping variable, with the five-group classification described above.

To assess how ecological factors may have influenced song variation, I used Mantel and partial Mantel tests to measure correlations among matrices representing geographic distance (the distance between recording sites), ecological distance (how different recording sites are with respect to climatic variables) and song distances (how different recordings are from each other with respect to song structure) between recordings. For ecological distance, I used climate data related to various aspects of rainfall and temperature; such data have been used by numerous other studies of geographic song variation (e.g., Ruegg et al., 2006b; Wei et al., 2015) in order to provide a broad picture of environmental effects on habitat. Climatic data representing the period 1950-2000, which matches well with the years of this study's recordings, were downloaded from http://worldclim.org, and 19 climatic variables representing temperature and precipitation data were extracted using ArcGIS (Esri, USA) for each recording site at a resolution of about 1 km^2. A principal component analysis was conducted in order to reduce the number of climatic variables. A matrix of the Euclidean ecological distances between each pair of recordings was calculated in SPSS using the principal components with eigenvalues over 1.00; likewise, a matrix of Euclidean song distances was calculated from the first two discriminant functions resulting from the discriminant function analysis. Using the longitude and latitude of each recording site, the package *fields* in the statistical analysis program R was used to calculate a matrix containing the great circle distance (the shortest route over the earth's surface between any two points) between each pair of recording locations.

The analysis program PASSaGE 2 (http://www.passagesoftware.net/) was used to conduct Mantel tests testing for correlations between each pair of distance matrices (song, geographic, and ecological), thus measuring the degree to which each pair (e.g., song structure and ecology) varied in concert. I also conducted partial Mantel tests, wherein a correlation between two matrices is calculated while a third is factored in to control for its influence.

5.3 RESULTS

5.3.1 Principal component analysis and initial classification

Analyses included recordings of 100 individuals, containing 873 song types and representing the three major geographic groups (and 10 different subspecies) (Figure 5.2). Examples of songs from each subspecies included are shown in Figure 5.3. A principal component analysis conducted to reduce the initial 20 acoustic variables generated five principal components with eigenvalues greater than 1.00 (see Table 5.2 for factor loadings). Together, these principal components explained 79.57% of the variation observed in the original 20 variables.

Because the principal components data did not meet the normality requirements for analysis using MANOVA and discriminant function analysis, I initially assessed the data using a classification method that does not require normal data distribution, k-nearest neighbor analysis. This approach correctly predicted the group membership of 89% (89/100) individuals. With the exception of one individual, all of the incorrect predictions arose from two sub-groups. In the case of *C.g. slevini*, a Western Lowland

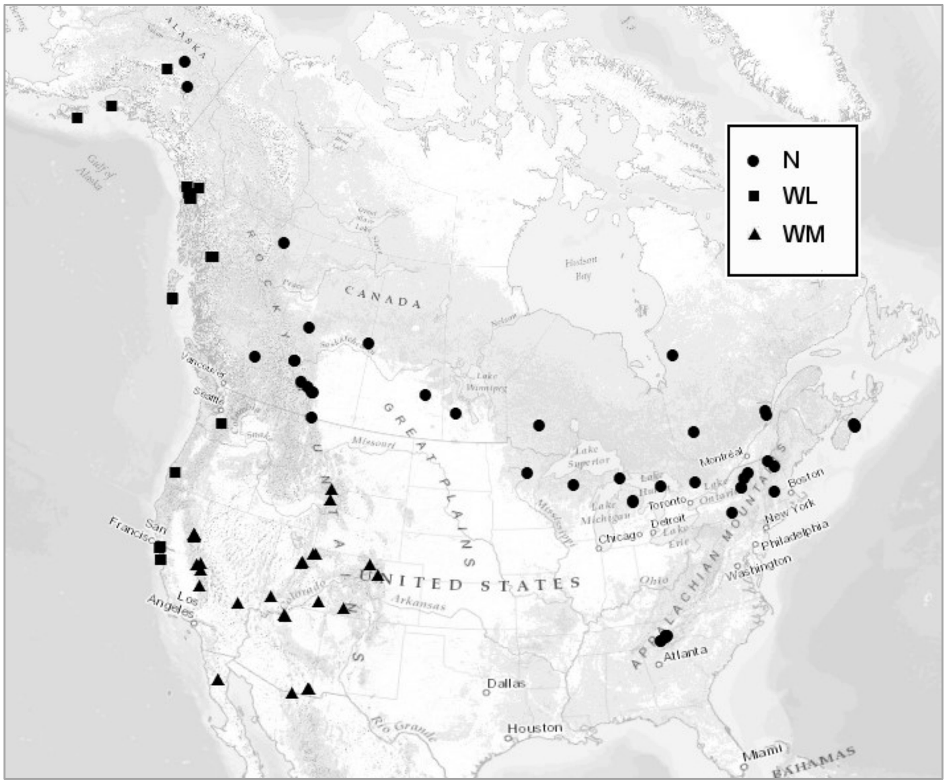

Figure 5.2 Map showing the locations of recordings analyzed in this study, divided into their major geographic groups of subspecies: Northern (N; n = 45), Western Lowland (WL; n = 23), and Western Mountain (WM; n = 32). Some symbols may be obscured in cases where separate recordings of a different

individual have the same or similar coordinates. Geographic group affiliation was determined using GPS coordinates, based on the descriptions of the ranges for the subspecies making up the groups, provided by Dellinger et al. (2012).

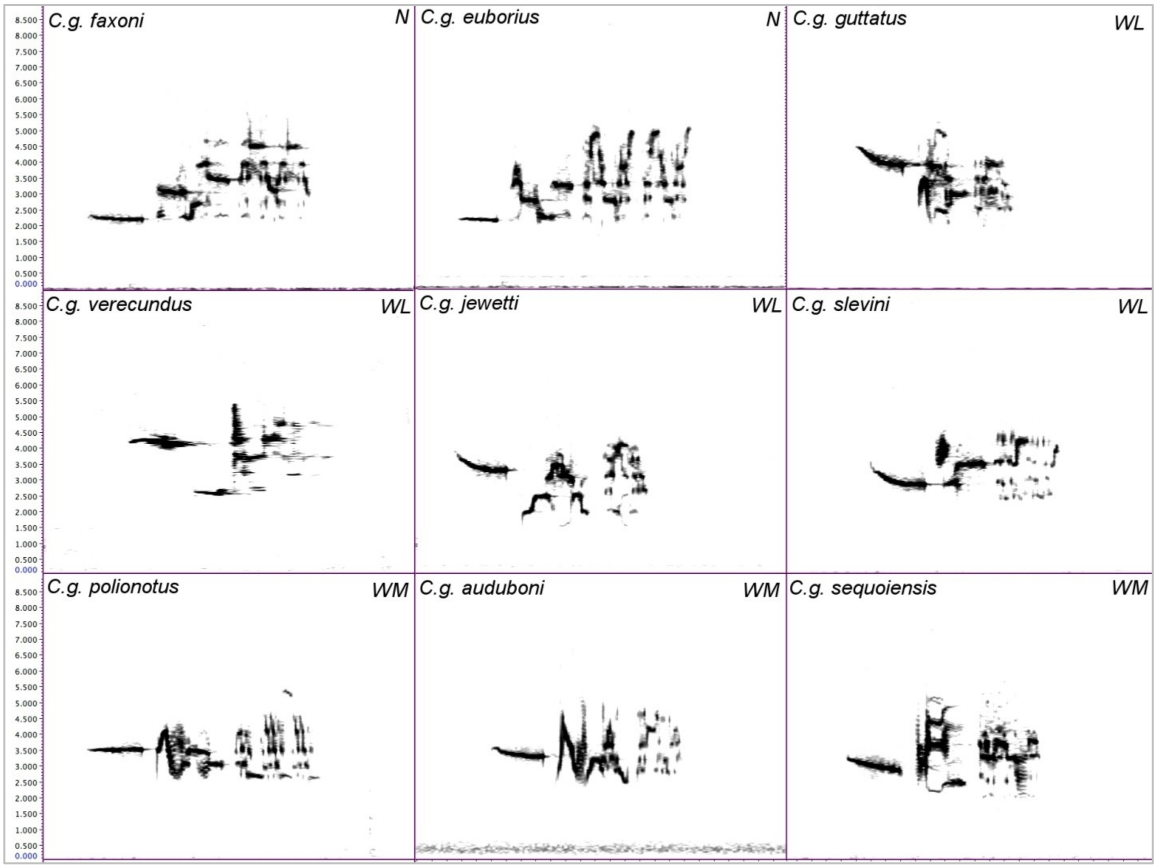

Figure 5.3 Spectrograms showing example song types from subspecies within the Northern (a-b), Western Lowland (c-g), and Western Mountain (h-j) groups.

subspecies, group membership was incorrectly predicted for five individuals. Among members of the Northern group subspecies *C.g. euborius*, six of the individuals included were recorded at high altitudes (> 1900 m) in the Canadian Rockies, whereas the other seven were recorded at lower altitudes (<1500 m) in the surrounding area; the analysis incorrectly predicted group membership of five of the six high-altitude individuals. On the basis of this preliminary analysis, the 100 birds were divided into five groups

Table 5.2 Factor loadings of acoustic variables for principal components with eigenvalues > 1. High factor loadings are in bold.

Variable	PC1	PC2	PC3	PC4	PC5
FI1$_{Mean}$	**.956**	.136	.185	.076	-.080
FI1$_{Max}$	.275	**.664**	.539	.158	-.011
FI1$_{Min}$	**.950**	-.168	-.037	.079	-.112
FI2$_{Mean}$	**.925**	.176	.238	.134	-.086
FI2$_{Max}$	.484	**.614**	.488	.131	.006
FI2$_{Min}$	**.934**	-.167	-.012	.125	-.130
FIΔ_{Mean}	**.833**	-.128	-.245	-.302	.168
FIΔ_{Max}	**.698**	-.084	-.194	-.383	.257
FIΔ_{Min}	.542	-.080	-.423	-.032	.195
FPMin$_{Mean}$	-.201	**.773**	-.030	.484	.105
FPMin$_{Max}$	-.309	**.861**	-.058	.144	-.041
FPMin$_{Min}$	.085	.111	-.281	**.812**	.281
FPBand$_{Mean}$	-.056	.214	**.907**	-.106	.163
FPBand$_{Max}$	-.050	.061	**.795**	-.061	.185
FPBand$_{Min}$	.012	.137	**.765**	-.163	.013
FPPeak$_{Mean}$	.060	**.781**	.419	-.004	.262
FPPeak$_{Max}$	-.068	**.852**	.166	-.239	.137
FPPeak$_{Min}$	.289	.123	.429	.149	**.594**
TI$_{Mean}$	.190	-.144	-.121	-.119	**-.680**
TP$_{Mean}$	-.483	.064	.502	.214	.034
Variance explained (%)	29.68	27.23	11.53	5.99	5.15

(Northern, Western Lowland, Western Mountain, high-altitude *C.g. euborius*, and *C.g. slevini*), which met the within-class normality requirements of the subsequent analyses. The "high-altitude *C.g. euborius*" group consisted of the six Northern (*C.g. euborius*) individuals located at high elevations (> 1900 m) within the Canadian Rockies.

5.3.2 Comparison between groups

A MANOVA was conducted using the principal components in order to compare song structure across the five groups (Northern, Western Lowland, and Western Mountain, with *C.g. slevini* and high-altitude *C.g. euborius* treated separately). The analysis revealed an overall difference in song structure across the five groups (Pillai's Trace = 2.029, F = 19.351, $p < 0.001$). There were significant differences by group with respect to PC1 (F(4, 100) = 420.812, $p < 0.001$), PC2 (F(4, 100) = 22.851, $p < 0.001$), PC3 (F(4, 100) = 9.015, $p < 0.001$), and PC4 (F(4, 100) = 7.666, $p < 0.001$)), but not for PC5 (F(4, 100) = 1.864, $p < 0.073$). Individual data for PCs 1-4 are shown in Figure 5.4.

Introductory note frequencies were highest in Western Lowland birds and lowest in Northern birds. Bonferroni-corrected comparisons between groups revealed that PC1, with high loadings for the mean and minimum values of introductory note frequency (FI1 and FI2) and absolute frequency change within the introductory note (FIΔ) (see Table 5.2), was higher in the Western Lowland group than the Western Mountain group ($p < 0.001$), and higher in the Western Mountain group than the Northern group ($p < 0.001$) (Figure 5.5a). The introductory note frequencies of these groups occupied dramatically different ranges of values, as can be seen by plotting the introductory note frequencies of all song types measured (Figure 5.6). The introductory note frequencies of Northern birds span a large range (~ 1700-5000 Hz in terms of FI1), whereas those of Western Lowland (~4000-5500 Hz), and Western Mountain (~3000-4000 Hz) birds cover comparatively restricted ranges. Figure 5.5 also demonstrates the differences in frequency change within the introductory notes (FIΔ), the magnitude of which is reflected by the distance from the line representing no frequency change (slope = 1): FIΔ was largest in Western Lowland birds, smaller in Western Mountain birds, and very small in the Northern group. With respect to PC1, high-altitude *C.g. euborius* birds were intermediate between Western Lowland and Western Mountain individuals, while *C.g. slevini* birds were most similar to the Western Mountain group.

Post-introductory song frequencies were higher in Northern and Western Lowland birds compared to Western Mountain birds. For both PC2 and PC3 (Figures 5.5b and 5.5c), most strongly associated with post-introductory frequency measures (the mean and maximum values for FPMin and FPPeak, as well as all FPBand values), the Northern group was higher than the Western Mountain group (PC2: $p < 0.001$; PC3: $p < 0.001$), as was the Western Lowland group (PC2: $p < 0.001$; PC2: $p = 0.001$). The Northern and Western Mountain groups did not differ from each other. High-altitude members of *C.g.*

euborius displayed lower PC2 values (FPMin and FPeak) than Northern (p = 0.003) and Western Lowland birds (p = 0.005), and did not differ from any groups with respect to

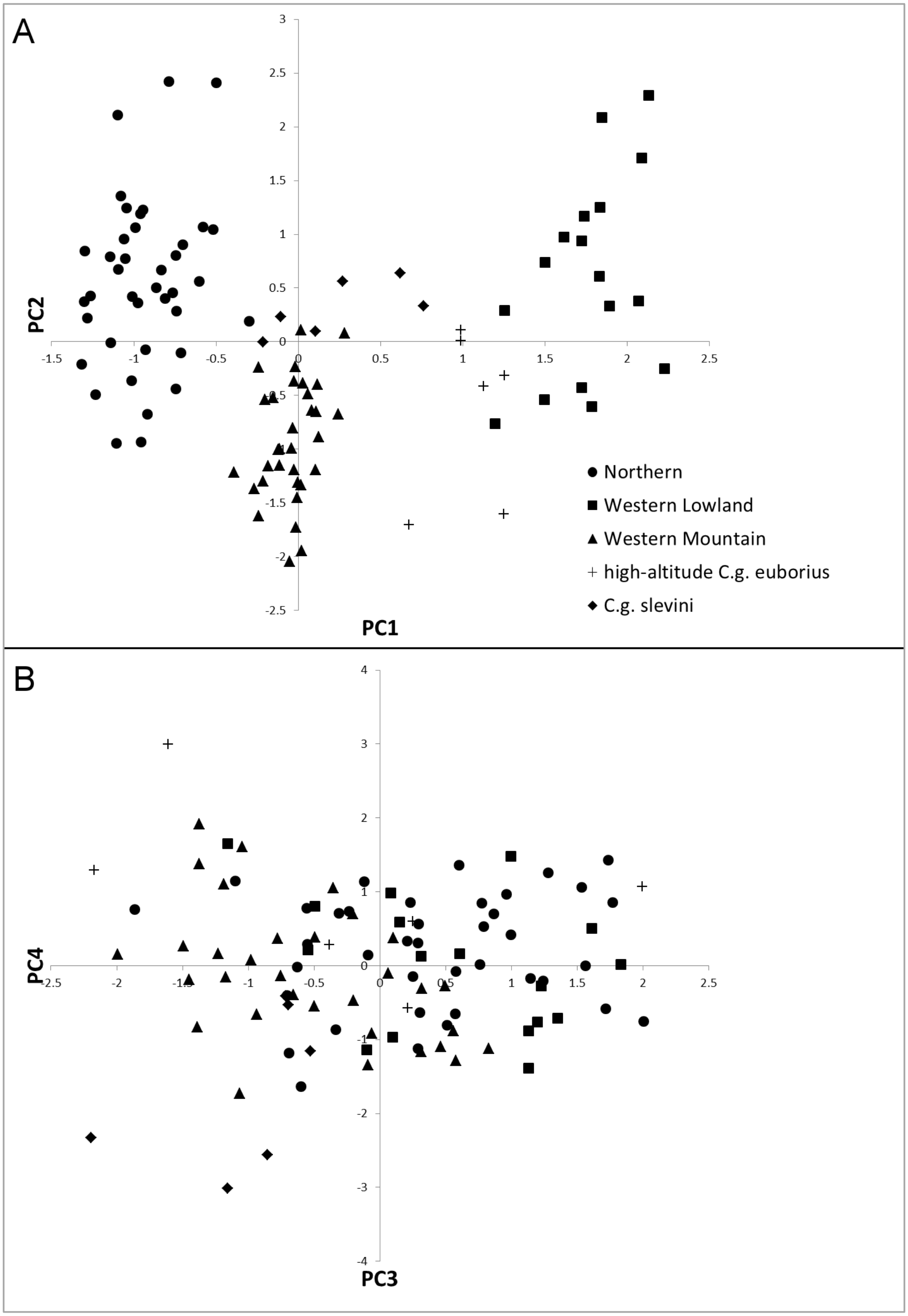

Figure 5.4 Scatterplots showing individual data for PCs 1 and 2 (A) and PCs 3 and 4 (B).

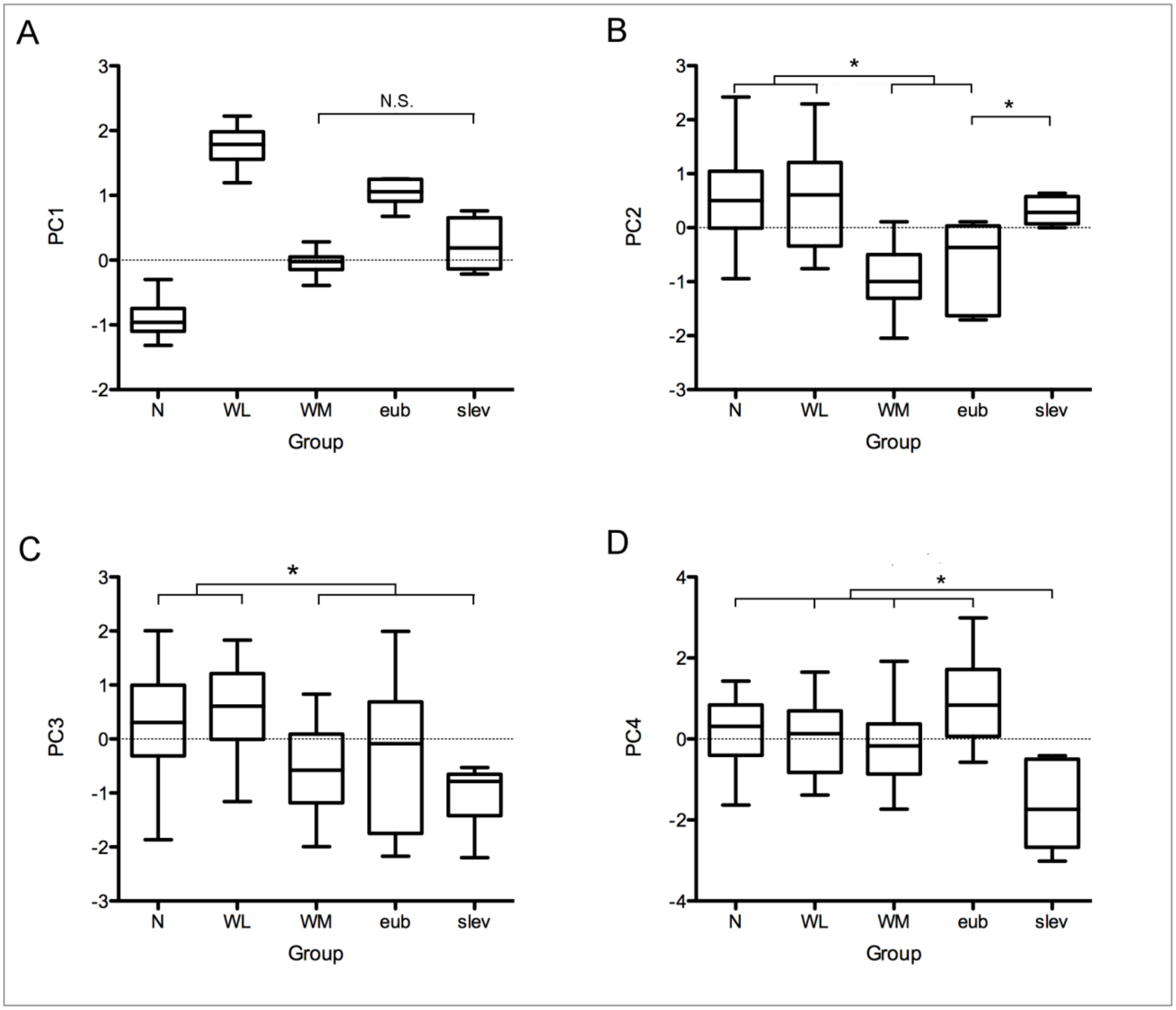

Figure 5.5 Boxplots showing group data for PCs 1-4 (A-D). Significant differences between groups (Bonferroni-corrected post-hoc comparisons; $\alpha = 0.05$) is indicated by asterisks (*); for PC1, all between-group differences were significant except for one (marked by "N.S.").

PC3 (FBand). *C.g. slevini* birds were lower than Northern ($p = 0.003$) and Western Lowland birds ($p = 0.002$) with respect to PC3, but similar to those groups with respect to PC2 (Fig. 3.5b and 3.5c). Values for PC4, associated with minimum post-introductory peak frequency ($\text{FPPeak}_{\text{Min}}$) and introductory note duration (TI), were significantly lower in *C.g. slevini* compared to the other groups ($p < 0.003$ for each) (Figure 5.5d).

Because neither of the temporal characteristics loaded highly on PCs that explained substantial variation in the original variables, each was assessed separately via one-way ANOVA. Duration of both the introductory note (TI) and post-introductory song portion (TP) differed across the groups (TI: F(4,95) = 8.011, p < 0.001; TP: F(4,95) = 11.917, p < 0.001) (Figure 5.7). Bonferroni-corrected post-hoc comparisons revealed that TI was greater in Western Mountain birds compared to Northern birds; in contrast, TP was greater in Northern birds than in Western Lowland and Western Mountain birds. High-altitude *C.g. euborius* birds did not differ from the rest of the Northern group with regard to either temporal characteristic, nor did *C.g. slevini* birds differ from the rest of the Western Lowland group in these respects.

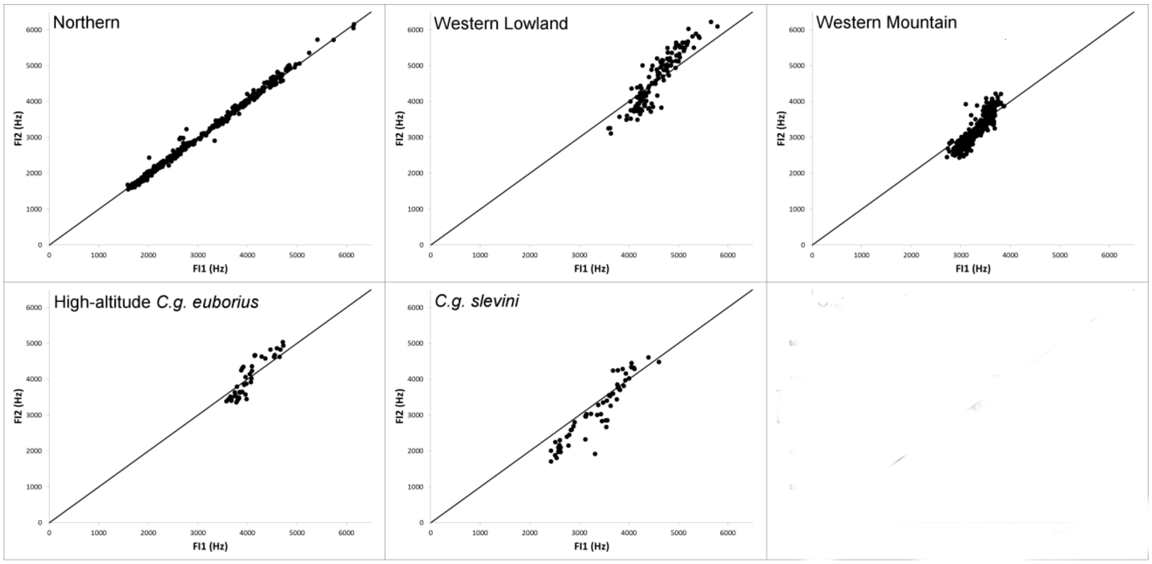

Figure 5.6 Scatterplots showing FI1 and FI2 for all song types of individuals from the Northern (n = 369 song types), Western Lowland (n = 129), and Western Mountain (n = 270) groups. Song types from high-altitude (>1900 m) C.g. euborius individuals from the Northern group (n = 45) and birds from *C.g. slevini* (n = 60) are shown separately. Diagonal lines indicate where FI1 and FI2 would be equal, representing no change in frequency within the introductory note (FIΔ = 0); thus, distance from the lines reflect the magnitude of FIΔ.

5.3.3 Discriminant function analysis

To see how accurately the 100 individuals could be distinguished from each other on the basis of song structure, a discriminant function analysis (DFA) was conducted using the five principal components and geographic group (Northern, Western Lowland, and Western Mountain, plus *C.g. slevini* and high-altitude *C.g. euborius* in their own groups) as the grouping variable. The results further demonstrated that song structure was distinctly different between the groups, as the DFA correctly classified 97.0% (97/100) of the individuals (Figure 5.8). The three incorrectly classified individuals included a *C.g. slevini* bird misclassified as Western Mountain, a Western Mountain bird misclassified as *C.g. slevini*, and a Western Lowland bird misclassified as high-altitude *C.g. euborius* (see filled-in symbols in Figure 5.7).

The position of group centroids and the distribution of individuals indicated that song structures across the three major geographic groups (Northern, Western Lowland, and Western Mountain) were highly distinct from each other, with Northern and Western Lowland song structure more similar to each other than either was to the Western Mountain group (Figure 5.8). *C.g. slevini* song structure was similar to that of the Western Mountain group (this similarity accounted for two of the three misclassifications), while structure in high-altitude *C.g. euborius* was intermediate between the Western Lowland and Western Mountain groups.

5.3.4 Relationships between song structure, geographic distance, and ecological factors

To examine possible connections between ecology and song structure, I conducted Mantel tests designed to measure the correlations between differences in song structure, geographic distance, and ecology across individuals and their recording locations.

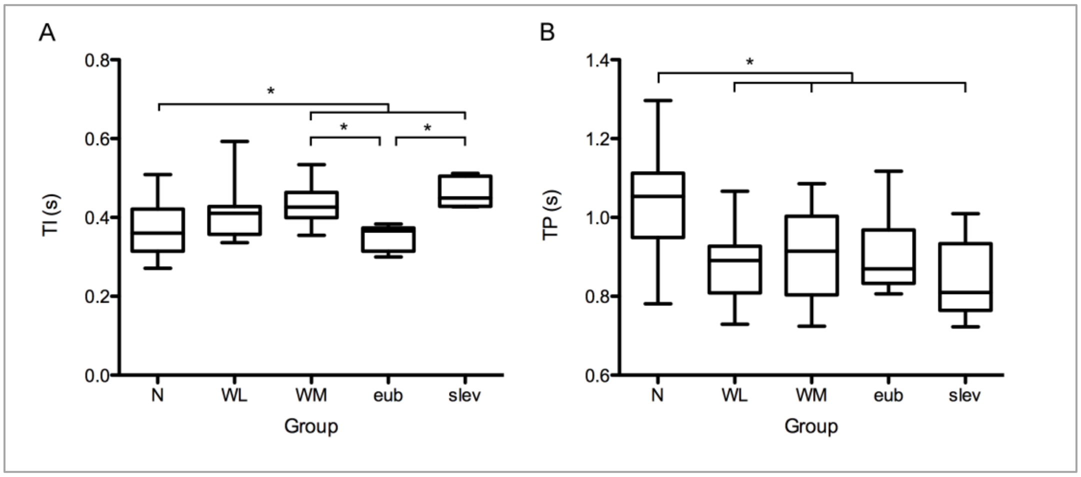

Figure 5.7 Boxplots showing group data for duration of the introductory note (A) and the post-introductory song portion (B). Significant differences between groups (Bonferroni-corrected post-hoc comparisons; α = 0.05) is indicated by asterisks (*).

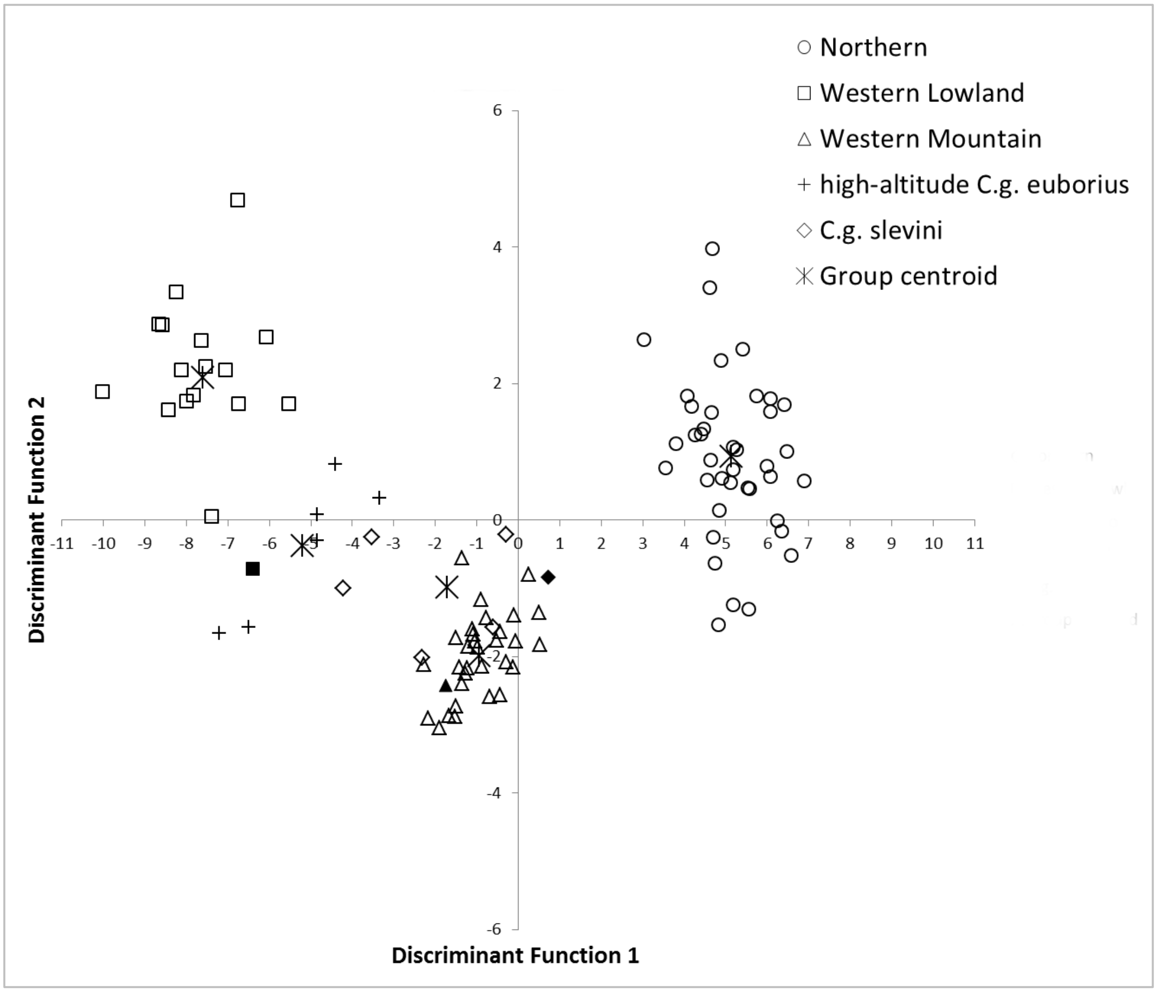

Figure 5.8 Plot of discriminant functions 1 and 2 representing results of discriminant function analysis. Symbols represent group affiliation; empty symbols represent correctly classified birds and filled-in symbols represent misclassified birds.

Ecological distance was assessed using 19 climatic variables representing data related to rainfall and temperature for each recording location (listed in Table 5.3). To reduce the number of climatic variables for use in analyses, I conducted a principal component analysis. This generated four PCs with eigenvalues above 1.00, which combined to explain 91.20% of the variation across recording sites (Table 5.3).

Mantel tests comparing song, ecological, and geographic distance matrices revealed significant correlations between Euclidean song distance and geographic distance ($r = 0.583$, $p < 0.001$) and between song distance and ecological distance ($r =$

Table 5.3 Bioclimatic variables and their factor loadings in principal component analysis.

Bioclimatic variable	PCClim1	PCClim2	PCClim3	PCClim4
Annual Mean Temperature	**.724**	.191	.000	**.643**
Mean Diurnal Range (Max Temp – Mean Temp)	.132	**-.801**	-.296	.216
Isothermality ((Var2/Var7)*100)	**.788**	-.199	-.444	.135
Temperature Seasonality (Standard deviation * 100)	**-.893**	-.300	.136	.153
Max Temp (Warmest Month)	.105	-.501	-.107	**.799**
Min Temp (Coldest Month)	**.884**	.372	-.078	.172
Temperature Annual Range (Var5-Var6)	**-.760**	-.552	.027	.185
Mean Temp (Wettest Quarter)	-.584	.090	-.064	**.633**
Mean Temp (Driest Quarter)	**.930**	.129	.017	-.044
Mean Temp (Warmest Quarter)	.090	-.078	.114	.951
Mean Temp (Coldest Quarter)	**.910**	.255	-.100	.253
Annual Precipitation	.310	**.759**	.530	-.005
Precipitation (Wettest Month)	.384	**.885**	.101	-.019
Precipitation (Driest Month)	.027	.297	**.942**	-.011
Precipitation Seasonality (Coefficient of variation)	.257	.285	**-.863**	.114
Precipitation (Wettest Quarter)	.385	**.877**	.122	-.012
Precipitation (Driest Quarter)	.053	.328	**.935**	-.005
Precipitation (Warmest Quarter)	-.286	.319	**.804**	.199
Precipitation (Coldest Quarter)	.582	**.716**	.121	-.060
Variance explained (%)	42.47	26.97	14.24	7.53

0.342 $p < 0.001$). When partial Mantel tests were conducted in order to measure correlations between two variables while excluding the third, song distance was still significantly correlated with both geographic distance ($r = 0.512$, $p = 0.001$) and ecological distance ($r = 0.117$, $p = 0.001$). Thus, the degree of song structure difference between individuals was associated both with the geographic distance between those individuals and with the degree of ecological differences between them, as calculated using climatic data.

To further explore relationships between song distance and climatic and geographic distances, I performed the same tests within the Northern, Western Lowland and Western Mountain group. In the Northern group, there was a significant correlation between song and geographic distance ($r = 0.187$, $p = 0.012$), which did not persist after controlling for ecological distance ($r = 0.045$, $p = 0.564$). There was also a significant correlation between song distance and ecological distance ($r = 0.209$, $p = 0.001$), and it remained after the influence of geographic distance was excluded via partial Mantel test ($r = 0.155$, $p = 0.014$). When I excluded the high-altitude *C.g. euborius* individuals and reran the tests, song distance was correlated with neither ecological nor geographic distance.

To examine potential ecological effects within the western lineage, I ran Mantel tests on pooled Western Lowland and Western Mountain birds. The test revealed a significant correlation between geographic and song distance ($r = 0.876$, $p < 0.001$), which persisted after controlling for ecological distance ($r = 0.840$, $p < 0.001$). There was also a significant correlation between ecological and song distance ($r = 0.477$, $p < 0.001$), but controlling for geographic distance decreased the strength of the association ($r = -0.021$, $p = 0.717$). In the Western Lowland group, there was a strong correlation between geographic and song distance ($r = 0.827$, $p < 0.001$), which persisted after controlling for ecological distance ($r = 0.625$, $p < 0.001$). There was a strong correlation between song and ecological distance ($r = 0.695$, $p < 0.001$), which did not persist after controlling for geographic distance ($r = -0.075$, $p = 0.505$). When I repeated the tests without *C.g. slevini,* there was a correlation between song distance and ecological distance ($r = 0.548$, $p = 0.002$), although it did not remain after exclusion of geographic distance ($r = 0.350$, $p = 0.032$). In the Western Mountain group, Mantel and partial Mantel tests revealed no significant correlations between song distance and either ecological or geographic distance.

5.4 DISCUSSION

This study examined geographic variation in hermit thrush song structure by analyzing the acoustic characteristics of songs from 100 individuals across the species' breeding range. Findings demonstrated that there are distinguishing differences in song structure between the three major geographic groups (Northern, Western Lowland, and Western Mountain). Analysis also revealed that there were some subgroups, *C.g. slevini* and high-altitude members of *C.g. euborius,* that differed structurally from their parent groups (Western Lowland and Northern, respectively). Underlining these differences, a discriminant function analysis based on 20 acoustic variables assigned individual birds to their respective geographic category with a high level of accuracy.

In terms of vocal acoustics, the geographic groups differed most dramatically in terms of introductory note frequency, which on average was highest in the Western Lowland group and lowest in the Northern group. The most distinct difference, however, related to the distribution of introductory note frequencies across all song types: while the introductory notes of Northern birds covered a large frequency range that spanned about 4000 Hz, the introductory notes of western birds each spanned a much smaller range of frequencies (~4000-5500 Hz for Western Lowland, ~3000-4000 Hz for Western Mountain). Among the western birds, Western Lowland introductory notes occupied a higher and somewhat larger range of frequencies than those of Western Mountain birds. Frequency change between the start and end of the introductory note was largest in Western Lowland birds and smallest in Northern birds.

Groups also differed on post-introductory frequencies, although these differences were less dramatic than those for introductory note frequencies. Whereas the distribution of introductory note frequencies was distinctive in each group, the post-introductory portion of song occupied similar frequency distributions across the groups (see Figure 5.5). Differences were nonetheless present, with the post-introductory portion of the song reaching higher frequencies and exhibiting a greater frequency bandwidth in the Northern and Western Lowland groups compared to the Western Mountain groups. Temporal characteristics also differed across groups, as Western Lowland and Western Mountain songs had longer introductory notes, but shorter post-introductory portions, than Northern songs.

5.4.1 Evolutionary divergence of major geographic groups

Genetically, hermit thrush can be divided into two distinct groups representing eastern (stretching west to eastern BC, roughly corresponding to the Northern group) and western (corresponding to the Western Lowland/Western Mountain groups) populations (Kerr et al., 2007; Topp et al., 2013; Alvarado et al., 2014) that also differ from each other morphologically (mainly with respect to body size; Alvarado et al., 2014). Detailed genetic analysis, together with comparisons of morphology and migratory routes, suggest that these two groups diverged following separation by ice sheets approximately one million years ago (Alvarado et al., 201; Weir and Schluter, 2004).

While this study's analysis of song structure identified three distinct clusters that aligned well with the three major geographic groups, some aspects of the variation between birds observed in this study nonetheless align with the documented division into eastern and western lineages. Overall song structure was more similar between the Western Lowland and Western Mountain groups compared to the Northern group (see DFA results; Figure 5.8). And the largest difference in acoustic variables was between the Northern group and the Western Lowland/Western Mountain groups, both for introductory note frequency and for temporal characteristics (i.e., duration of the introductory and post-introductory parts of the song).

Thus, some of the variation in hermit thrush song structure may have its origins in the initial separation into eastern and western lineages. Although secondary contact between the eastern and western groups was re-established during the period following initial separation, isolation of the two groups has been maintained by a migratory divide and considerable differences in migratory routes (e.g., eastern birds migrating to eastern British Columbia take an indirect route that goes north into central Canada before traveling west) and overwintering grounds (Alvarado et al., 2014). Genetic hybrids within the migratory divide, or hybrid zone, are thought to experience decreased reproductive fitness due to intermediate migratory routes that lead to low return rates, thus contributing to maintenance of the divide and continued isolation of the eastern and western groups

(Alvarado et al., 2014). Within these isolated eastern and western lineages, there is some validity to the idea that some aspects of song structure likely diverged due to genetic differences, and possibly cultural drift associated with song learning.

Alvarado et al. (2014) found that birds within the hybrid zone were intermediate with respect to genetics and body size. Only three of the birds analyzed in this study fell within the hybrid zone identified by Alvarado et al. (2014), and all of them were correctly classified by the DFA. The high probability of correct classification (> 98%) for two of these individuals suggests that their song structures were not intermediate but rather very similar to the groups they are affiliated with. In contrast, the low probability of correctness for the third bird (~50%) suggests that, in terms of song structure, it does not fall as clearly into any of the groups. Further recording will clarify whether birds in the hybrid zone have song structure that is intermediate, reflecting a mix of the song characteristics belonging to the birds on either side; the alternative, seen in Swainson's thrush individuals located in a similar hybrid zone (Ruegg et al., 2006b), is that each bird has songs that are not intermediate but rather like one group or the other.

Some of the song structure differences within the two groups of western lineage (i.e., between the Western Lowland and Western Mountain groups) may be related in part to further separation events. In examining the evolutionary histories of boreal superspecies, including sapsuckers (*Sphyrapicus* spp.) and subspecies of fox sparrow (*Passerella iliaca*), Weir and Schluter (2004) pointed not just to the separation event 1 million years ago but also to a second one, which involved more southern glacial advances that occurred around 700 000 years ago. For the superspecies complexes discussed by Weir and Schluter (2004), this second period of separation led to division of the western groups into so-called Pacific Coast and Rocky Mountain fragments, which geographically align with this study's Western Lowland and Western Mountain groups, respectively. While there is no evidence of further genetic divergence within the western lineage of hermit thrush, it is possible that this second separation event contributed to differences between the two western groups. Any song divergence that accumulated (e.g., via cultural drift) may have been maintained in part by the group's different migration patterns: Western Lowland birds travel north up the Pacific Coast, while Western

Mountain birds are altitudinal migrants that winter in valleys near their Rocky Mountain breeding grounds (Dellinger et al., 2012).

5.4.2 Morphological and functional reasons for the differences

Frequency differences between the two western groups may relate in part to body size, which has been negatively correlated with song frequency in a number of songbird species (Ryan and Brenowitz, 1985). The frequency of both introductory and post-introductory song components was higher in Western Lowland birds, a group reported by Aldrich (1968) to contain the smallest of all hermit thrush subspecies, than in Western Mountain birds, which were the largest. This variation in body size may also contribute to the comparatively larger bandwidth of the post-introductory portion of song in Western Lowland birds, as bandwidth has also been negatively correlated with body size in some species (e.g., Mason and Burns, 2015).

Notably, the nature and degree of the variation observed often differed between the introductory and post-introductory portions of song. For example, introductory note frequencies were highest in Western Lowland birds and lowest in Northern birds, and occupied dramatically different ranges of the spectrum; in contrast, post-introductory frequencies of Western Lowland and Northern birds were similar to each other and higher than those of Western Mountain birds. Similarly, temporal characteristics of the two song components exhibited different relationships across the groups; for example, Western Lowland and Western Mountain birds had the longest introductory notes, whereas Northern birds possessed the longest post-introductory song durations. These differences in the magnitude and direction of variation suggest that the two components of hermit thrush song have, to some degree, been shaped independently, possibly subject to different influences.

One potential reason for this “disconnect” is that the introductory and post-introductory parts of the song serve different functions and, as a result, are intended to travel different distances, a phenomenon documented in some other songbirds. For example, in blackbirds (*Turdus merula*), the song’s whistled introductory portion is associated with long-range species recognition by males and females whereas the more complex portions of the song, which are more vulnerable to degradation and therefore do not travel as far, are important for individual recognition by nearby males (Dabelsteen and Pederson, 1993; Dabelsteen et al., 1993). In this and other species (e.g., Aubin et al., 2014; Cosens and Falls, 1984), the song forms or song parameters most resistant to degradation are the ones associated with long-range species recognition.

Since hermit thrush have a comparable song structure, with a whistled introductory note and complex post introductory portion, it may be that a similar functional division exists, with the introductory note serving in long-range species recognition (possibly directed mainly at females) and the rest of the song more associated with short-range individual recognition by neighbouring males. Based on playback experiments with rufous-sided towhees (*Pipilo erythrophthalmus*), Richards (1981) characterized that species' introductory song portion as an alerting component. He suggested that, because it is more resistant to degradation than other, more complex song components, the introduction allows for basic conspecific recognition at a distance, upon which the listener may approach in order to assess specific components of the more complex post-introductory portion (possibly related to individual recognition) at closer range.

While no studies have examined the transmission qualities of hermit thrush song (e.g., how transmission through a forest habitat effects each song component), other research shows that whistles of consistent frequency are more resistant to degradation than other types of song elements (Brown and Handford, 2000) and that their transmission may even be aided by forest reverberations (Slabbekoorn and Smith, 2002b). The introductory note of hermit thrush song may be similarly influenced by its forest habitat.

Research in another thrush species, the veery (*Catharus fuscescens*), suggests that its introductory note is more involved in long-range communication than territorial interactions. Playback experiments indicate that its song's introductory note is not essential for eliciting territorial responses (McNally et al., 1986), and the low-amplitude soft songs that male veeries use in close-range territorial interactions lack introductory notes (Belinsky et al., 2015). Like veeries, hermit thrush males often sing songs lacking an introductory note in response to territorial intrusions (see next chapter). The absence of the introductory note in an apparent short-range, aggressive signal by hermit thrush supports the idea that the introductory portion functions primarily for long-range signaling. If this is the case, the introductory note may be subject to unique influences, such as sexual selection. Further playback studies, in combination with transmission experiments, will provide further information on the respective roles of the introductory and post-introductory portions of hermit thrush song. This, in turn, will help in understanding the forces that have shaped each part of the signal.

5.4.3 Role of altitude and habitat

Overall, song structure was relatively consistent within each of the major geographic groups. One interesting exception was the Northern subspecies *C.g. euborius*, in whom song structure was unlike other Northern group birds and intermediate between Western Lowland and Western Mountain structure, as indicated by their DFA misclassification (Figure 5.8). Compared to other members of the Northern group, these birds had songs with higher introductory note frequencies and lower post-introductory frequencies.

It is possible that environmental factors at different elevations contributed to song differences between the misclassified individuals and the rest of the Northern group. Whereas the other *C.g. euborius* individuals were located below 1500 m, the six high-altitude birds were recorded at elevations ranging between 1910 and 2275 m, an elevation at which the habitat becomes more open as trees such as Engelmann spruce (*Picea engelmannii*), subalpine fir (*Albies lasiocarpa*), and lodgepole pine (*Pinus contorta*) give way to shrubs like mountain heathers as the sub-alpine forest transitions towards the alpine tundra (Luckman, 1990). Particularly for the introductory note, which may function in long-range communication and therefore be more subject to the habitat's effects on sound transmission, this open habitat may have led to comparatively higher frequencies, as predicted by the acoustic adaptation hypothesis (Wiley and Richards, 1978).

Divergence in song structure along an elevation gradient has been documented in some other songbird species, such as the grey-breasted wood-wren (*Henicorhina leucophrys*), in which song frequency increased gradually over a similar altitude range to the one examined in this study (Caro et al., 2009). Due to the limited number and coverage of the recording sites examined here, it remains unclear whether song structure differs gradually with elevation, as in other species (e.g., Caro et al., 2009) or changes more abruptly at a certain elevation (e.g., where habitat transitions to open tundra). Recording individuals at various altitudes along this elevation gradient, possibly in conjunction with playback experiments to examine responsiveness to different song forms, will clarify the nature and significance of song structure variation in this part of the species range.

As with *C.g. euborius*, members of *C.g. slevini*, a Western Lowland subspecies, were frequently misclassified by the DFA, indicating that their song structure differs from the rest of the Western Lowland group. While there were differences in temporal characteristics, both the introductory and post-introductory portions of the song were lower in frequency among *C.g. slevini* individuals compared to other Western Lowland birds. It may be that these differences relate to differences in habitat: *C.g. slevini*, located mainly along the California coast, occupies the Mediterranean California ecoregion, while other Western Lowland members are located within the Marine West Coast Forests ecoregion. Whereas the Mediterranean California ecoregion is characterized by shrubland and exhibits seasonal variability in precipitation, the Marine West Coast Forests ecoregion is a rain forest characterized by the highest levels of precipitation in North America.

While the correlation observed between song distance and ecological distance suggests that environmental factors drive song variation to some degree, the song structure differences between groups argue against a prominent role for habitat. In particular, birds in the Western Mountain group, which occupy open, high-altitude habitats, sang at the lowest frequencies – opposite of what would be predicted by the acoustic adaptation hypothesis (Wiley and Richards, 1978). It may be that the association between environment and song structure is somewhat spurious: other characteristics of the birds and their habitats, not accounted for by the Mantel tests, may actually drive the observed differences in song structure. For example, ecological differences between regions may covary with other factors that influence song divergence via cultural drift following separation of populations, natural selection's influences on morphology, and so on. Nonetheless, it may be that ecological differences have exerted stronger effects on song structure in some areas compared to others, as in *C.g. euborius* individuals at high altitude, where the terrain is so starkly different.

Overall, the observed patterns of geographic song variation in hermit thrush are likely the result of a complex mix of factors that have influenced song structure at various points during the species' evolutionary history. Most broadly, the most distinctive differences between the Northern and the Western Lowland/Western Mountain groups (e.g., introductory note frequency) likely date back to the separation of hermit thrush into eastern and western lineages, arising via genetic differences and cultural drift. Song divergence within the western lineage may also have resulted from cultural drift, maintained by differences in migration and overwintering between the Western Lowland and Western Mountain groups; it is also possible secondary consequences of natural selection's effects upon body size have contributed to spectral differences between these groups. Selection for optimal sound transmission in different habitats has also likely contributed to song variation, especially in the case of within-group variation (i.e., *C.g. slevini* and high-altitude *C.g. euborius*).

5.4.4 Conclusion

In summary, this study has documented reliable differences in song structure across the breeding range of hermit thrush. These geographic song differences parallel established genetic and morphological differences in some respects, although variation within and between the geographic groups highlight more extensive influences on song structure. Future work that examines song structure, morphology and genetics in concert – especially in areas of interest such as the hybrid zone identified by Alvarado et al. (2014) and the Canadian Rockies – will help to clarify the evolutionary history of hermit thrush as well as the forces that have shaped its song over that span. Such information has the potential to add to our overall understanding of how song structure variation contributes to speciation and associated processes in songbirds.

CHAPTER 6: TERRITORIAL RESPONSES TO PLAYBACK OF LOCAL AND FOREIGN SONG BY HERMIT THRUSH (*CATHARUS GUTTATUS*) MALES

6.1 INTRODUCTION

As discussed in the previous chapter, songbird species can exhibit substantial geographic variation with respect to song structure, most often resulting as by-products of evolutionary processes that lead to isolation of populations and, in some cases, speciation (Podos and Warren, 2007). Such song divergence may result from a number of causes, including differing effects of habitat upon sound transmission (Boncoraglio and Saino, 2007), the secondary consequence of natural selection acting on physical attributes related to song production (e.g., beak size and shape) (e.g., Huber and Podos, 2006), and new song patterns that accumulate in populations via errors during song learning (Lachlan and Servedio, 2004).

While most often a by-product of reproductive isolation and speciation, geographic song variation can also contribute to such processes because of the centrally important role that song plays in songbird breeding behaviour. Along with territorial defense, song is used by male songbirds to attract females for mating (Catchpole and Slater, 2003). Indeed, females assess male quality and make mating choices based on elements of song (Catchpole, 1987; Searcy and Yasukawa, 1996). A prerequisite to such assessment and selection is recognition of the singer as a member of the same species (i.e., conspecific recognition), as signalled vocally by certain song characteristics that vary across songbird species. As geographic song divergence progresses, females may no longer recognize males with songs that have diverged sufficiently from such species norms, leading to assortative mating and reproductive isolation of groups within a species (e.g., subspecies) (Price, 2008). Such a lack of conspecific recognition, and its consequences related to mating and speciation, has been documented in a number of songbird species, including gray-cheeked thrush (*Catharus minimus*; Ouellet, 1993) and greenish warbler (*Phyloscopus trochiloides;* Irwin et al., 2001).

The question of conspecific recognition is of particular relevance in hermit thrush (*Catharus guttatus*), given recent evidence of genetic divergence during the species' evolutionary past. Indeed, several studies have documented substantial genetic differences between eastern and western lineages of hermit thrush (Kerr et al., 2007; Topp et al., 2013; Alvarado et al., 2014), reflecting a division likely initiated by glacial advances one million years ago and maintained despite secondary contact by a migratory divide occupied by hybrids of compromised reproductive fitness (Alvarado et al., 2014). This genetic divergence raises the possibility of separation of hermit thrush into separate species, as has occurred in some other superspecies complexes with similar evolutionary histories (Weir and Schluter, 2004).

There are substantial geographic differences in song structure that, to a good degree, mirror the genetic split between eastern and western lineages. Rivers and Kroodsma (2000) found frequency and temporal differences between songs from two distant populations, one western (Arizona) and one eastern (New England): eastern songs had introductory notes that were shorter and lower in frequency, but post-introductory portions that were higher and longer (Figure 6.1). The previous chapter elaborated on these findings, showing that some of the structural differences (temporal characteristics, introductory note frequencies) were relatively consistent within the lineages. Nonetheless, there were some aspects of song that differed considerably within each lineage; for example, within the western lineage, birds from the two western groups,

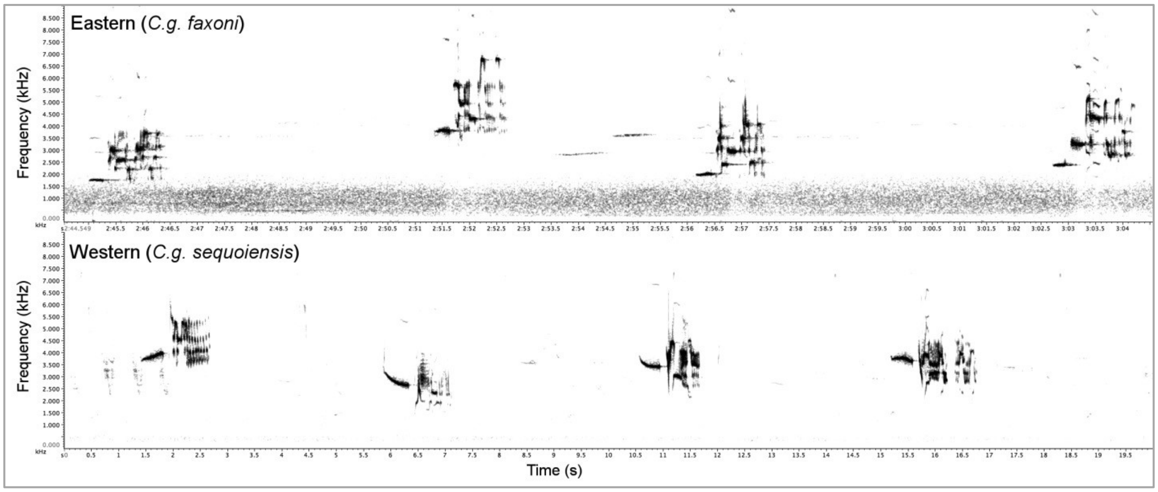

Figure 6.1 Representative spectrogram examples of eastern and western playback song structure.

Western Lowland and Western Mountain, differed significantly in terms of both introductory and post-introductory song frequency. Such within-lineage differences indicate that, following the initial split into eastern and western lineages, other factors (e.g., cultural drift, morphological differences, and habitat's effect on sound transmission) may have led to further divergence in song structure.

Given the genetic differences between hermit thrush lineages, which may signify eventual division into separate species, as well as the extensive geographic song differences that align with this split, information on how territorial males respond to local versus foreign songs (i.e., songs from each lineage) can provide valuable information on conspecific recognition. Such information has the potential to add to our understanding of the species' evolutionary past, as well as its current and future states.

Although species recognition by female songbirds is most pertinent to questions related to song's role in evolutionary processes, studies on the topic are most often conducted using male subjects. One reason is that it is methodologically easier to study males, whose aggressive responses to territorial playback can be assessed in the field. In contrast, female responses to song variation are most often studied in the lab, requiring capture, captivity, and, in some cases, estradiol treatment (e.g., Searcy et al., 1997; 2002). Females can be studied using playback in the field but is more difficult due to females being harder to locate on territories (e.g., Brambilla et al., 2008). The behavioural responses of a male songbird to territorial intrusion simulated by playback are robust and easy to measure; they include increased proximity, flights, and investigation and attack of the sound source (e.g., Liu et al., 2008; Colbeck et al., 2010). Males exhibit a stronger territorial response to local songs (e.g., songs of the same subspecies) than to songs from more distant populations in a number of species, including swamp sparrows (*Melospiza georgiana*; Liu et al., 2008), white-crowned sparrows (*Zonotrichia leucophrys*; Derryberry, 2011), and dark-eyed juncos (*Junco hyemalis*; Reichard, 2014).

In addition to aggressive physical responses, numerous aspects of vocal behaviour have been at one time or another associated with male territorial defense. These include changes in singing rate and in the rate of song type switching (how frequently a bird switches between song types), as well as song overlapping (cutting off the other bird's song) and song type matching (responding to a bird's song with the same song type) (Searcy and Beacher, 2009; Searcy et al., 2014). Studies have produced mixed results: studies in some species but not others have associated each of these behaviours with territorial aggression (for review, see Searcy and Beacher, 2009). These mixed results may not be surprising considering the aggressive vocal signals fail to meet all three of Searcy and Beacher's (2009) criteria for classification as an aggressive signal: the signal increases in occurrence during aggressive interactions; it prompts a physical response from the territorial male that suggests it is an aggressive signal; and, its use accurately predicts physical escalation of the conflict. One partial exception is song type matching, which appears to function as an honest aggressive signal during the early stage of the hierarchical, escalating system of signaling between song sparrow males. While playback experiments with western song sparrows supported this function (Ackay et al., 2013), a study with an eastern population did not (Searcy et al., 2013).

Much of the recent literature on aggressive signaling in songbirds has focused on the use of vocalization types associated with territorial conflict and not observed in other contexts (e.g., spontaneous singing not associated with conflict). The most common of these, soft songs (sometimes also referred to as "whisper songs"), are characterized by their low amplitude compared to the broadcast songs that a bird more commonly uses during singing, and are thus suited to the close-range communication that may unfold between males during territorial conflict (Titus, 1998; Anderson, 2007). Playback studies have shown that soft song meets the above-described criteria for classification as an aggressive signal (Searcy et al., 2014), and, unlike other purported aggressive signals (e.g., overlapping), it has been shown to be a reliable predictor of attack in multiple species, including swamp sparrows (Ballentine et al., 2008), song sparrows (*Melospiza melodia*; Ackay et al., 2011), and black-throated blue warblers (*Dendroica caerulescens*; Hof and Hazlett,2008).

In terms of song structure, the degree to which a bird's soft songs resemble its broadcast songs varies across species. In some songbirds, such as song sparrows and swamp sparrows, soft songs consist of both low-amplitude versions of broadcast songs and structurally unique vocalizations not seen in the bird's repertoire of broadcast songs (Anderson et al., 2008; Ballentine et al., 2008). In other cases soft songs are entirely unique vocalizations used only in that context (e.g., brownish-flanked bush warbler (*Cettia fortipes*); Xia et al., 2013). Songbirds may also exhibit vocalizations during aggressive conflict that are unique from their regular broadcast songs in other ways. For example, a recent study with veeries (*Catharus fuscescens*) documented use of songs distinguished not by their amplitudes but by the absence of a typical broadcast song component, the introductory note (Belinsky et al., 2015). Overall, the number of songbird species in which soft songs and other unique, possibly aggressive vocalizations have been examined, both with respect to form and function, remains small.

The main objective of this study was to compare the territorial response of hermit thrush males to local songs and to songs from a distant subspecies. To accomplish this, I conducted a playback study with a within-subjects design, comparing the response of eastern hermit thrush (*C.g. faxoni*) male subjects to territorial intrusion as simulated using playback recordings of broadcast songs from local birds as well as birds from a distant, western subspecies (*C.g. sequoiensis*). Based on the distinctive differences between eastern and western songs, I anticipated that males would respond more strongly to local songs of their own eastern subspecies compared to songs from a distant western subspecies. Additionally, aggressive signaling in hermit thrush males has not been studied systematically, and playback experimentation can provide valuable information on their vocal behaviour in such contexts, including matching, overlapping, and the use of unique vocalizations, whether characterized by amplitude (as in soft songs) or other attributes. Specifically I was interested in whether hermit thrush produced soft songs, or any other distinctive vocalization, associated with territorial aggression.

6.2 METHODS

The playback experiments were conducted within the Halifax Regional Municipality during the period between 12 June and 09 July, 2012. Subjects were 15 males from a local breeding hermit thrush population. Because there is no evidence that neighbours within this population share song types (Jones, 2005; Chapter 4; Roach, unpublished data), individuals could be reliably identified by their song types. Some of the subjects in this study had been color banded during the previous season, as part of another study; in those cases, individual identification was aided by visualization of colored leg bands.

6.2.1 Playback Recordings

Eastern playback stimuli consisted of recordings of eastern hermit thrush broadcast songs, captured from the local *C.g. faxoni* population and western playback stimuli consisting of recordings of broadcast songs of individuals from the western subspecies *C.g. sequoiensis* (Figure 6.1). For eastern birds, the playback stimuli were recorded by the authors within the Halifax Regional Municipality earlier during the same breeding season as playback using a Marantz PMD 670 solid-state digital recorder (PCM, 44.1 kHz, 16 bit) with a Sennheiser ME66 directional microphone. To minimize the possibility that subjects would have heard the playback individual before, in each case the location of a subject and the individual in the playback recording were several kilometres apart. Given the combined influences of moderate breeding site fidelity (35%; Schlossberg, 2009) and year-to-year mortality, it is unlikely that subjects had previous familiarity with the eastern playback songs that they heard.

For western playback, recordings were acquired from the Cornell Lab of Ornithology's MacAulay Library (macaulaylibrary.org). To achieve a similar level of within-group consistency as the eastern recordings, all of the western recordings were of individuals located in the Sierra Nevada Mountains in California (*C.g. sequoiensis*). *C.g. sequoiensis* was chosen because it represents a distant subspecies with distinctly different song structure (Figure 6.1; previous chapter), and also because there were a sufficient number of clear recordings available from a discrete area.

Specific recordings from each population were selected for use as playback based on recording quality, as well as featuring a single singing hermit thrush male and minimal background noise interference from other songbirds, wind, etc. Three-minute sections were selected from the original recordings using the audio editing software Audacity 1.3.12. Three minutes was chosen as the length of playback based on pilot playback sessions that indicated a fast and strong response to territorial playback, combined with the objective of not stressing the subjects more than necessary by eliciting a prolonged aggressive response. In each case, a high-pass frequency filter was applied in order to reduce noise at low frequencies (below the frequency range of the birds' songs), in order to eliminate background noises (e.g., traffic) that might fluctuate over the course of the playback recording. Singing rate within the playback recordings was kept as consistent as possible: the number of songs during the 3 minute playback recordings ranged from 39-41 in eastern playback stimuli and 38-40 in western playback stimuli. Playback recordings were loaded onto an IPod Nano for broadcast via field speaker (Saul Mineroff Electronics, Inc.).

6.2.2 Playback Procedure

All playback sessions were conducted between 05:00 and 08:00 AST. The order of playback was randomly counterbalanced across subjects, such that the first playback session would be eastern playback for approximately half of the subjects and western playback for the other half. (Among the 13 birds eventually used in analysis, seven were exposed first to eastern playback and then to western playback.) In order to minimize short-term influences of the first playback session on each bird's responses during the second session, while also minimizing the possibility that the bird's breeding status would not change between sessions, the second session was conducted two days after the first (except one case, in which the second session was delayed until four days after the first due to inclement weather).

A playback session was initiated when a known territorial male was found singing on his territory. An arena of radius 8m was set out quickly using ropes with flag markers; the location of the arena was selected based on previous observation of the bird with the objective of delineating an arena that was within the bird's territory but did not include its initial location. A field speaker was placed at the center of the arena on a raised platform, such that it was approximately 1m above the ground; the speaker's volume was set to a level that would produce playback at an amplitude of approximately 80 dB SPL, measured at a distance of 1m using a decimeter (Extech Instruments, model #407750). A wooden carving of a hermit thrush was attached to a nearby tree branch within a foot above the speaker in order to provide the subject with a visual focal point during playback.

After three minutes of recording spontaneous singing by the subject during a pre-playback period, the three-minute broadcast of the playback recording was initiated. During playback, the experimenters, positioned in secluded spots at the outer edge of the arena, narrated the bird's location in relation to the sound source as well as the occurrence of flights. In addition to these recording devices, a Marantz PMD 670 solid-state digital recorder, with a Sennheiser ME66 directional microphone, was placed near the center of the arena in order to help capture the subject's vocalizations. After the conclusion of playback, the experimenters continued to record the bird's vocal and physical behaviour for three additional minutes.

If the experimenters lost track of the subject, the playback session was aborted. Likewise, the playback session was aborted if there were physical responses and/or entry into the arena by any other hermit thrush male or female. Two males were excluded for these reasons, leaving 13 complete playback sessions for analysis.

6.2.3 Physical Responses to Playback

Data on the physical responses of birds during the three-minute playback session were extracted from the experimenters' recorded narration of playback session. A volunteer familiar with hermit thrush song but blind to the details of the playback session (i.e., identity of the playback recordings, pairing of the sessions) transcribed the narration with respect to location of the bird and the occurrence of flights. In total, six variables were measured: the average distance from the speaker (measured at 10 second intervals), closest approach to the speaker, time spent within a 5m radius of the speaker, the latency to approach within 5m of the speaker, the number of flights that passed within a 5m radius of the speaker, and the latency to the first such flight. The distance- and time-related narration of the experimenters was consistently the same or very similar to each other (e.g., within 1-2m for distance estimates). When different distance or time estimates were recorded by the two narrators, the average of the reported values was used. For latency-related variables, a value of 180 seconds (representing the total length of the playback session) was used in cases in which the bird did not enter the 5m radius or perform any flights. For distance-related variables, a value of 8m (representing the outer limit of the marked arena) was used for all distances that equaled or exceeded that distance.

6.2.4 Vocal Responses to Playback

Vocal responses of territorial males were examined spectrographically using RavenPro1.4. For each bird, a song type repertoire was established based on singing during the pre-playback period, as well as previously collected recordings of spontaneous singing for that male. The song type repertoires of birds used as eastern playback sources were also established by examining the playback recording in conjunction with additional recordings of each male. The sequence of song types delivered by the focal male before, during, and after playback was identified via visual examination of the spectrogram and comparison to the bird's known song type repertoire. Across the 26 playback sessions (two for each of 13 males), there were three cases (one to eastern playback and two to western playback) in which the subject delivered none or very few (less than 10) songs during the playback period. These three cases were excluded from the overlapping and matching analyses because they contained insufficient numbers of samples for accurately calculating rates of overlapping and matching.

Overlapping by playback subjects was identified by examining spectrograms of the playback sessions using RavenPro 1.4. An overlapping song was defined as a song that began before the end of a playback song. For each playback session, the observed rate of overlapping was calculated as the proportion of subject songs that overlapped a playback song. The rate of overlapping expected by chance was calculated using Ficken's (1974) method, which establishes the rate by comparing the observed number of overlaps to what would be expected if the subject was singing with no regard for the timing of the playback stimulus. In each case, the expected rate of overlapping was equal to the playback recording's duty cycle, or the proportion of the total playback time taken up by playback vocalizations; this was calculated by dividing the sum of the playback song durations by the total length of the recording (180 seconds).

Song type matching is not possible in hermit thrush because they share no or very few song types between repertoires (Jones, 2005; Chapter 4). However, males may match conspecific singers by singing a song type from its repertoire that is of a similar frequency; this sort of frequency matching has been observed in some other songbirds (e.g., Harris' Sparrow; Shackleton et al., 1991). Here, frequency matching was assessed in terms of high and low frequency song types, as the song types in the repertoire of each male within this population can be divided into such categories on the basis of absolute frequency (Chapter 4).

Frequency matching was assessed by examining spectrograms of the playback session. Songs by the subject were considered to be matching if they were of the same category (high or low song type) as the playback song that preceded it. Subject songs that began before the preceding playback song was complete were excluded, as were songs that did not immediately follow a playback song (i.e., if it was the second subject song since the previous playback song). This was to ensure the playback song was heard in its entirety by the subject bird before it responded. The rate of frequency matching for a given playback session was calculated as the proportion of the eligible songs that matched the preceding playback song. The expected matching rate for a session was calculated using the probabilities of high and low songs being used, calculated from the proportion of each song category observed during pre-playback. Thus, each eligible song during playback had an expected rate that reflected how frequently that song category was typically used (e.g., the expected probability for a high song type would be the probability of that bird singing a high song type). The overall expected rate of matching was calculated as the average of the expected probabilities of the eligible songs during playback.

To enable comparison across individuals and sessions, normalized rates of overlapping and frequency matching were calculated. In each case, the normalized rate was calculated by dividing the observed rate by the expected rate. Thus, values of greater than 1.00, 1.00, and less than 1.00, would represent overlapping (or matching) above chance, at chance, or below chance levels, respectively.

6.2.5 Statistical Analysis

Data related to subject responses to playback were analyzed using paired comparisons. Count data (e.g., number of flights) was compared using a non-parametric test, the

Wilcoxon signed rank test; for continuous variables, normally distributed data was compared using paired-sample T-tests, while non-normally distributed data was compared using Wilcoxon signed rank tests. Pearson correlations were used to assess the association between physical and vocal responses to playback.

6.3 RESULTS

6.3.1 Physical responses to playback

Overall, subjects demonstrated a stronger territorial response to eastern playback featuring songs from the same subspecies compared to western playback composed of songs from a distant, foreign subspecies (Figure 6.2). Males exhibited a closer average distance from the sound source (Wilcoxon Signed Rank Test: Z = -2.353, p = 0.019) and performed more flights within 5m of the speaker (Wilcoxon Signed Rank Test: Z = -2.415, p = 0.016) during Eastern playback; there was no difference between playback types with respect to closest approach to the sound source (Wilcoxon Signed Rank Test: Z = -1.736, p = 0.083). Males also spent more of the playback period within 5m of the sound source (Wilcoxon Signed Rank Test: Z = -2.429, p = 0.015), approached within 5m of the sound source earlier following onset of playback (Wilcoxon Signed Rank Test: Z = -2.668, p = 0.008), and performed their first flights sooner after the onset of playback (Wilcoxon Signed Rank Test: Z = -2.934, p = 0.003) in response to eastern playback.

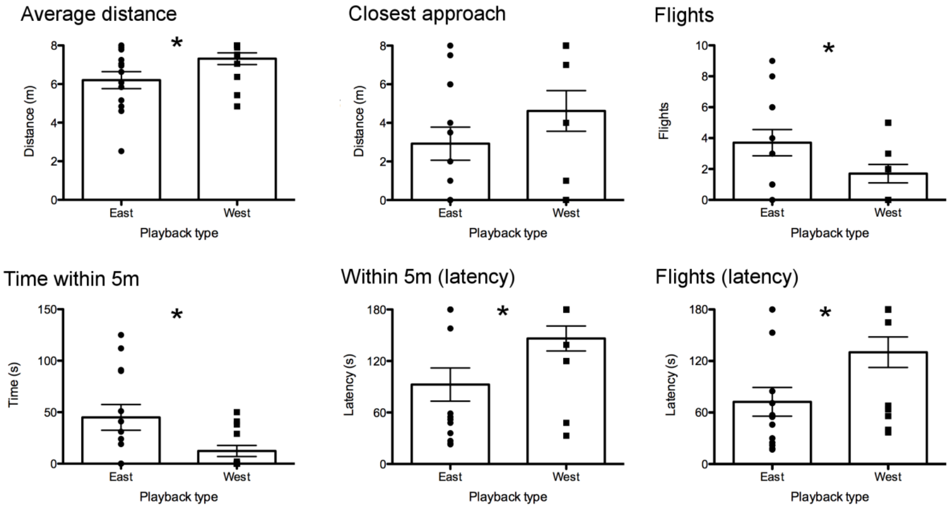

Figure 6.2 Plots of physical response variables in response for eastern and western playback, with bars representing mean values and error bars showing SEM. The individual data points for each subject are also shown (n = 13). Asterisks indicate statistically significant differences beween responses to eastern and western playback.

A principal component analysis was conducted using the six variables associated with physical responses to playback. Two principal components with eigenvalues greater than 1.00 resulted: PC1 was associated with the bird's proximity to the sound source and PC2 was associated with the bird's flights within 5m of the sound source (PC2) (Table 6.1). In total, these two principal components explained 86.07% of the variation in the original variables. As with the individual variables, a paired comparison revealed that birds exhibited a stronger territorial response during eastern playback than to western playback, (PC1, Wilcoxon Signed Rank Test: Z = -2.271, p = 0.023; PC2, Wilcoxon Signed Rank Test: Z = -2.621, p = 0.009). There was no effect of playback order on the physical responses to playback, as responses did not differ between the first and second playback session with respect to either PC1 (Wilcoxon Signed Rank Test: Z = -0.314, p = 0.753) or PC2 (Wilcoxon Signed Rank Test: Z = -1.363, p = 0.173).

6.3.2 Vocal behaviour in response to playback

Overall, birds vocalized similar amounts during eastern and western playback sessions. Paired comparisons revealed that the total number of songs (with all types of songs pooled together) did not differ between playback types before (Wilcoxon Signed Rank Test: Z = -0.118, p = 0.906), during (Wilcoxon Signed Rank Test: Z = -0.275, p = 0.784) or after playback (Wilcoxon Signed Rank Test: Z = -0.035, p = 0.972) (Figure 6.3). Regardless of playback type, the overall amount of singing decreased from the pre-playback to the playback period (Wilcoxon Signed Rank Test: Z = -2.140, p = 0.032) and

Table 6.1 Factor loadings of physical response variables for principal components with eigenvalues > 1.00. High loadings are highlighted in bold.

Variable	PC1	PC2
Distance from speaker	**.933**	.109
Closest approach to speaker	**.880**	.077
Time within 5m of speaker	**-.952**	.023
Within 5m of speaker (latency)	**.828**	.326
Flights (within 5m)	-.082	**-.958**
Flights (latency)	.125	**.928**
% of total variance explained	58.26	27.81

then did not differ from playback to post-playback (Wilcoxon Signed Rank Test: Z = -0.143, p = 0.886) (Figure 6.3).

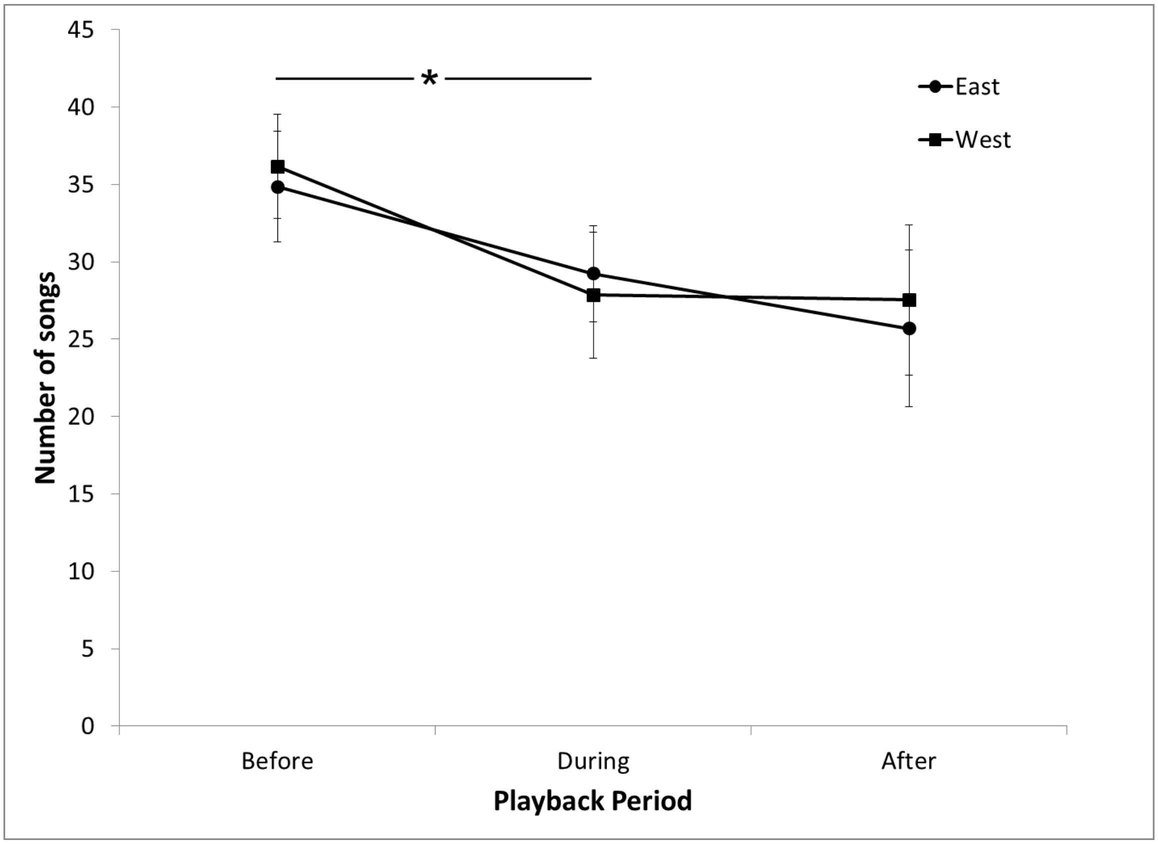

Figure 6.3 Mean number of songs delivered during eastern and western playback sessions. Error bars represent SEM. Asterisk indicates a significant difference in the amount of singing between playback periods.

Abridged songs

During and after playback, some subjects sang not only broadcast songs from their standard song type repertoires but also songs that were characterized by their lack of introductory notes (Figure 6.4). These songs, which I will hereafter refer to as “abridged” to reflect their lack of introductory notes, were not observed during pre-playback, nor had I observed them during previous spontaneous singing by the subjects in this study. The majority of the abridged songs (202 of 231, or 87.4%) were versions of broadcast songs from the bird’s normal repertoire. The others were novel abridged songs that were structurally distinct from the bird’s repertoire of broadcast songs; these also lacked introductory notes (Figure 6.4). Table 6.2 summarizes the use of abridged broadcast and novel song types by the nine subjects that sang abridged songs. Among these subjects, the number of novel song types observed among abridged songs was correlated with neither subject repertoire size (r = 0.175, n = 9, p = 0.653) nor the number of songs analyzed for repertoire size calculation (r = 0.356, n = 9, p = 0.347).

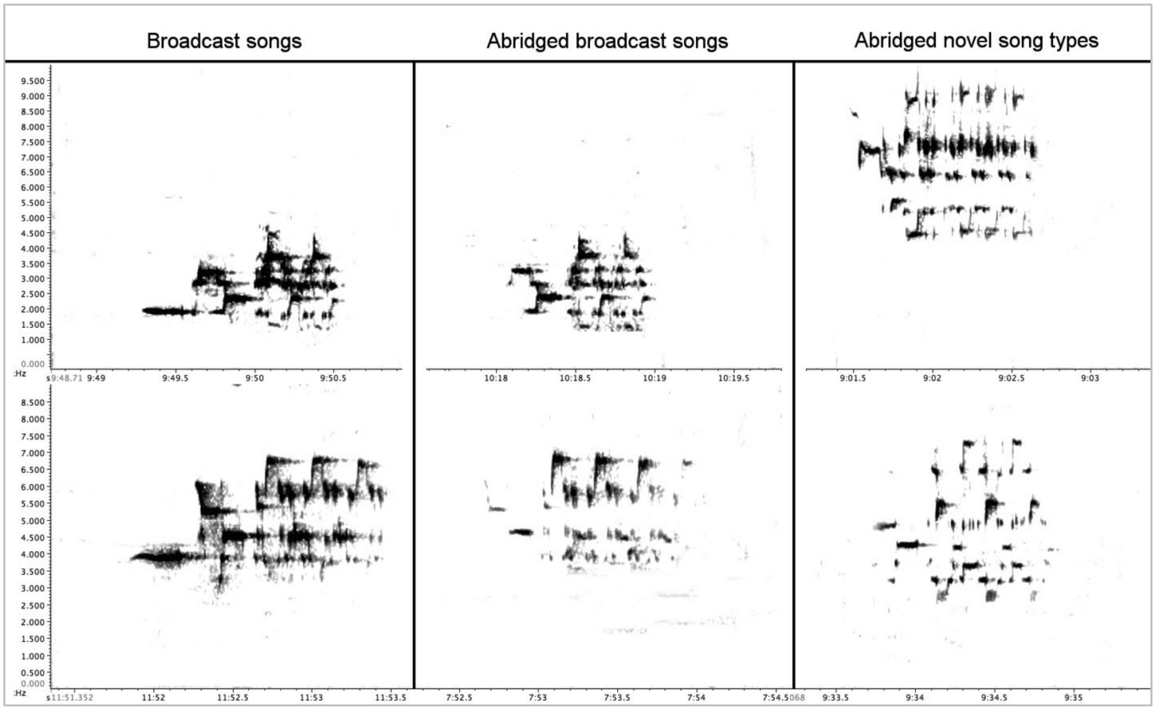

Figure 6.4 Examples of broadcast songs, abridged broadcast songs, and abridged novel song types from two hermit thrush males (one in each row).

Table 6.2 Broadcast song repertoire size, the number of songs recorded for repertoire size calculation (including previous recordings of spontaneous singing), and the number of distinct abridged broadcast and novel songs for subjects that sang abridged songs.

Subject	Broadcast song repertoire size	Number of songs analyzed	Abridged broadcast song types	Abridged novel song types
1	8	508	7	1
2	12	506	4	1
3	12	471	9	2
8	12	221	9	0
9	11	962	4	2
10	9	244	6	1
11	8	330	9	1
12	7	933	3	0
13	8	246	7	1

Figure 6.5 shows how abridged songs were used in response to eastern and western playback. The number of abridged songs was greater in response to eastern playback than to western playback (Wilcoxon Signed Rank Test: $Z = -2.668$, $p = 0.008$). Likewise, the latency to first use of soft song was significantly shorter during eastern playback compared to western (Wilcoxon Signed Rank Test: $Z = -2.666$, $p = 0.008$).

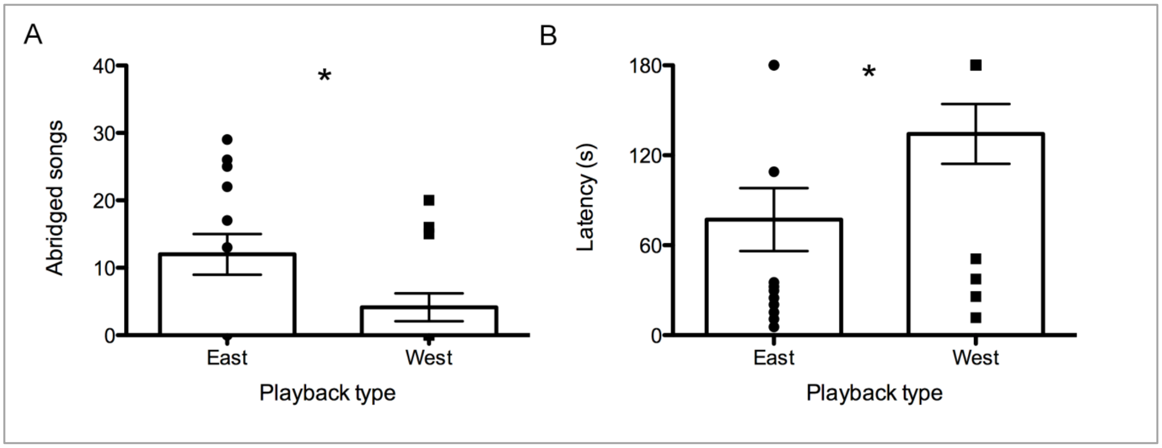

Figure 6.5 Mean total number of abridged songs (A) and mean latency to onset of abridged songs (A) during eastern and western playback. Error bars represent SEM. Individual data points for subjects are also shown (n = 13). Asterisks represent significant differences between eastern and western playback.

There were no abridged songs observed prior to playback, and a small number were heard during the three-minute post playback period (a total of 21 from five subjects, compared to a total of 210 during playback periods). The number of abridged songs used during playback was significantly correlated with the bird's proximity to and approach towards the sound source (PC1; $r = -0.881$, $n = 26$, $p < 0.001$), but not with flight activity (PC2; $r = -0.338$, $n = 26$, $p = 0.091$).

Song overlapping and frequency matching

Song overlapping occurred at rates significantly below chance levels during both eastern (expected proportion: 0.310 ± 0.018; paired-samples T test: $t(11) = 3.639$, $p = 0.004$) and western (expected proportion: 0.287 ± 0.024; paired-samples T test: $t(10) = 7.517$, $p < 0.001$), indicating in both cases that territorial males tended to sing in a way that avoided overlapping the playback recordings. Comparison of normalized overlapping rates revealed a statistical trend towards birds overlapping more in response to eastern playback than to western playback (paired-samples T test: $t(10) = 2.126$, $p = 0.059$).

Overall, the normalized rate of overlapping did not approach chance levels (1.00) in any cases except for the eastern playback sessions of three birds, for which the overlapping rates were 1.06, 1.08, and 1.12 (indicative of overlapping at slightly above-chance levels). Normalized rate of overlap was significantly correlated with proximity to the sound source (PC1; r = -0.682, n = 23, p < 0.001) but not with flights (PC2; r = -0.101, n = 23, p = 0.646).

During eastern playback, occurrence of frequency matching was significantly lower than would be expected by chance (expected proportion: 0.533 ± 0.050) (paired-samples T tests: p(11) = 7.127, p < 0.001). That is, birds avoided matching, typically responding to a given playback song with one from the opposite category (high or low) (see Figure 6). Normalized rate of frequency matching was not significantly correlated with either of the PCs representing physical responses to playback (PC1: r = -0.007, n = 12, p = 0.982; PC2: r = 0.037, n = 12, p = 0.908), indicating that magnitude of physical response was not associated with level of frequency matching.

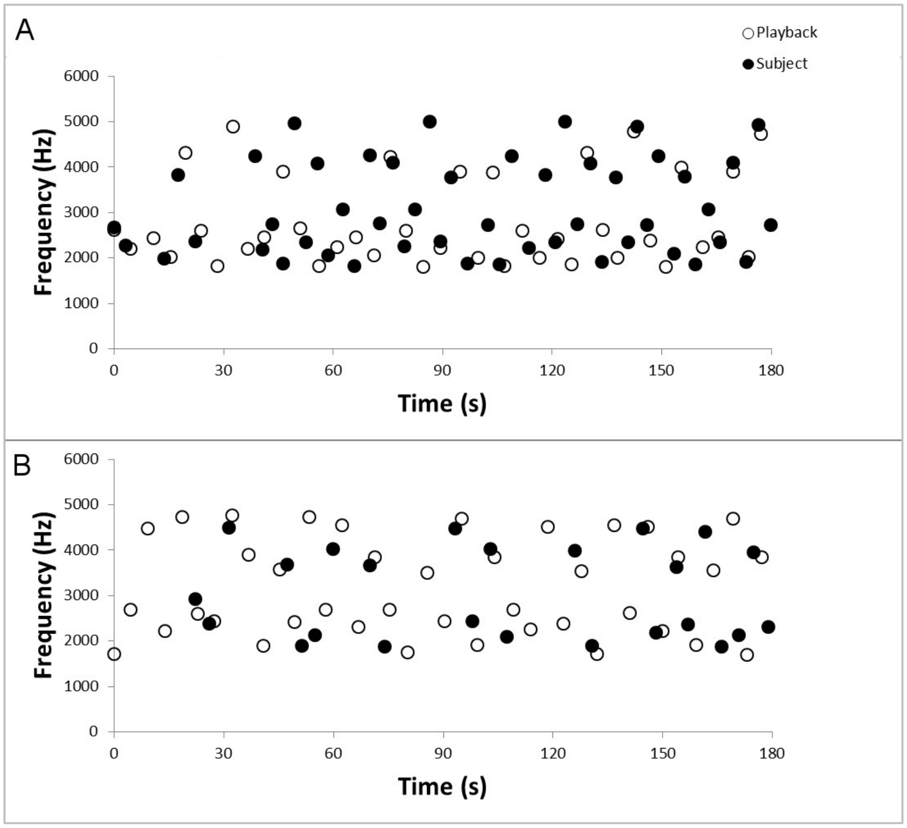

Figure 6.6 Examples of territorial male vocal interactions with eastern playback recordings, including a bird that frequency matched (high or low song types) at approximately chance levels (A; normalized matching rate = 1.05) and one that matched at below-chance levels (B; normalized matching rate = 0.22).

6.4 DISCUSSION

The main finding of this study was that hermit thrush males show a stronger territorial response to playback of songs from their own, local population than to songs from another, distant subspecies. In response to eastern playback, members of the eastern subspecies *C.g. faxoni* approached the sound source earlier, spent more time close to it, and did more investigative flights in the area of the sound source in response to locally recorded sound playback. The territorial response was expressed in the use of abridged broadcast vocalizations that lacked introductory notes as well as song types that were not part of the broadcast repertoire. In addition, I demonstrated that territorial males avoid overlapping of song playback and also avoid frequency matching by responding to playback songs with song types from their repertoire that occupy the opposite portion of the song's frequency spectrum.

6.4.1 Differential Recognition

The fact that males in this eastern population exhibited a stronger territorial response to songs from their own subspecies compared to songs from another, distant subspecies suggests that, at a minimum, they are able to discriminate local from non-local songs. The degree to which this reflects an overall ability to discriminate between subspecies based on song is difficult to conclude from the present data, since the songs from the same subspecies were recorded from the local area. Although they were recorded far enough away that the subjects were very unlikely to have been previously exposed to them, it is unclear the degree to which the discrimination observed represents differences between local versus foreign dialects, between different subspecies, or both. Given previous evidence of structural song differences, however minor, between different populations within the same subspecies (Chapter 4), it may be productive to compare responses to playback from local and non-local populations of the same subspecies in order to clarify the nature of differences in species recognition. Nonetheless, the present data indicate a difference in recognition of local versus non-local songs.

The differences in response strength were not unexpected given the extensive structural song differences between the eastern (*C.g. faxoni*, a Northern group subspecies) and western (*C.g. sequoiensis*, a Western Mountain subspecies) playback groups. As the previous chapter details, the songs of *C.g. faxoni* exhibit a much wider range of introductory note frequencies than those of *C.g. sequoiensis*. Less dramatic but nonetheless significant differences also exist with respect to frequency of the post-introductory portion and duration of both song components. In terms of which song characteristics were used for discrimination between playback types, it is possible that male responses differed on the basis of introductory note frequency, especially if introductory notes serve an attention-capturing role related to conspecific recognition, as has been suggested in other species (Richards, 1981). Males may also have discriminated on the basis of differences that have not been analyzed bioacoustically, such as the organization or characteristics of notes within the post-introductory song portion (see Figure 6.1). Studies in other songbirds have demonstrated that differential response to playback can depend on subtle differences such as note order (e.g., Liu et al., 2008).

The question of song discrimination is of particular interest in hermit thrush, for which recent research has demonstrated a genetic divergence into eastern and western lineages with differences that raise the possibility of separate species status (Kerr et al., 2007; Topp et al., 2013; Alvarado et al., 2014). While these results suggest that males can discriminate between these two lineages based on song, it would be interesting to examine female discrimination, since it is the influence of their discrimination upon mate choice that potentially contributes to reproductive isolation and, potentially, speciation. Studies in various songbirds have demonstrated sex differences in responsiveness to local versus non-local songs, most often with females exhibiting more selective discrimination (e.g., Searcy and Brenowitz, 1988; Searcy et al., 2002). Examining female preferences for local versus foreign songs in hermit thrush, whether in the field or the laboratory, would likely provide valuable insights into the evolutionary implications of song divergence within the species.

In understanding the functional implications of hermit thrush song divergence it will also be useful for future research to assess differential responsiveness in other populations and subspecies besides the one studied here. In some songbirds, such as the black-throated blue warbler and the gray-breasted wood wren, studies have found asymmetry in responses, wherein members of one subspecies showed discriminative responses while another did not (Colbeck et al., 2010; Dingle et al., 2010). Thus, the differences seen in this study's population may not be reflective of discrimination by other hermit thrush subspecies. Conducting playback studies in other parts of its breeding range will reveal whether hermit thrush subspecies show similar differences in species recognition, as subspecies do in some studies (e.g., Bombilla, 2008), or different, asymmetric responses, as they do in others (e.g., Colbeck et al., 2010). Playback experiments in the hybrid zone where both eastern and western lineages occur (Alvarado et al., 2014) may also provide valuable information on conspecific recognition. Together, such studies have the potential to provide valuable insights regarding recognition, mate choice, and, following from that, evolutionary processes within the species.

6.4.2 Use of Abridged Songs

In many cases, birds responded to territorial playback with songs lacking introductory notes, displaying a vocalization form that has not been documented in hermit thrush and has not been observed by the researchers except in one territorial skirmish between two neighbouring males recorded in a different neighbourhood within the Halifax Regional Municipality (personal observation, May, 2012). While the vast majority of these abridged broadcast songs were versions of song types from the bird's documented repertoire, in some cases the abridged songs did not match repertoire songs. In all such cases, the post-introductory song delivered resembled those of high song types in composition and absolute frequency. It is possible that these were actually repertoire song types that had not previously been documented, although this is unlikely because repertoires were established based on a large amount of spontaneous singing (from both the playback sessions and other recordings of the same individual) in most cases. The absence of a correlation between the number of novel song types observed and the number of songs analyzed for assessment of song type repertoires suggests that the novel songs may be used exclusively during aggressive interactions, rather than versions of broadcast song types that were not used during previously recorded spontaneous singing.

The observation of these abridged songs parallels recent findings by Belinsky et al. (2015), who observed songs without introductory notes used by male veeries in response to territorial playback. As in that study, the abridged songs here fulfilled one of

Searcy and Beecher's criteria for aggressive signals, the context criterion: songs without introductory notes were used exclusively in aggressive contexts and were not observed during pre-playback or previous recordings of the subjects (nor in extensive recordings of spontaneous singing by other territorial males). Although there was a strong association between use of abridged songs and proximity to the sound source, it was difficult to explicitly assess the predictive criterion because males did not directly attack the wooden mount. It is possible that doing more playback experiments using a more realistic taxidermic mount would generate attacks and allow for testing of this criterion. In this regard, veeries did not attack a taxidermic mount either (Belinsky et al., 2015). Lastly, Searcy and Beecher's response criterion, wherein playback of the signal (in this case, the abridged songs) prompts an aggressive response from the subject, was not tested in this study; in veeries, this criterion was tested but not met. Future experiments may address this criterion by using recordings of abridged songs as stimuli.

As a song form that has so far only been documented in aggressive contexts, these abridged songs may serve a similar function to that of low-amplitude songs in other songbird species (e.g., song sparrows), although the criteria for aggressive signals have not been met in this case as they have for soft songs (Searcy et al., 2014). If abridged songs fulfill an equivalent role in hermit thrush, the difference in song characteristics may relate to the functions of the song components. As discussed in the previous chapter, it may be that the introductory note functions primarily in long-range communication (e.g., conspecific recognition by potential mates), while the post-introductory portion serves a close-range role involving individual recognition between neighbouring males. Indeed, in veeries, the introductory note is not vital for eliciting territorial male responses (McNally et al., 1986). Thus, in both hermit thrush and veeries, males in aggressive contexts may adapt their singing by dropping the part destined for long-range signaling and employing only the part necessary for conspecific recognition. Future research may add to our understanding of the functional components of hermit thrush song by further examining the role of these abridged songs in aggressive contexts, possibly by employing abridged songs as playback stimuli.

6.4.3 Other Vocal Behaviours in Response to Playback

I assessed vocal responses to playback with respect to some other vocal behaviours that have previously been associated with territorial aggression. One of these is song overlapping, which has been proposed to be an aggressive signal by some researchers (e.g., Mennill and Ratcliffe, 2004); however, the overall evidence is not compelling and overlapping does not meet the criteria for aggressive signals (Searcy and Beacher, 2009). In this study, males consistently overlapped both eastern and western playback recordings at below-chance levels, indicating that they adjusted their temporal singing patterns to avoid overlapping. The normalized rate of overlapping was correlated with the bird's physical response to playback, in terms of position relative to the speaker (PC1), such that birds who exhibited a stronger response avoided overlapping to a lesser degree. Considering that the highest observed rates represented overlapping at chance, rather than above-chance, levels, this association likely does not reflect a role for overlapping as an aggressive vocal signal. The decreased avoidance of overlapping in birds showing a strong territorial response more likely reflects the shift in focus away from maintaining an active space for vocalizations and towards physical defense of territory.

Such avoidance of overlapping aligns with studies in songbirds other than chickadees, such as Eurasian wrens (*Troglodytes troglodytes*; Yang et al., 2014) and lesser skylarks (*Alauda gulgula*; Gochfeld, 1978), in whom singers actively adjusted the timing of their singing in order to avoid overlapping conspecifics. Songbirds often avoid temporal overlapping not just with songs of their own species but also with heterospecific birds; for example, Popp et al. (1985) showed that several forest songbird species, including the wood thrush (*Hylocichla mustelina*) avoid overlapping the songs of other species. Together, this research suggests that songbirds avoid overlapping of birdsong in general in order to maintain an active space for their own songs and increase the likelihood of being heard (Searcy and Beacher, 2009). Considering that singers avoid overlapping not just conspecific but also heterospecific songs in some songbird species, it would be interesting to include an additional playback group in future playback experiments that features recordings of a heterospecific songbird with similar singing behaviour, such as Swainson's thrush (*Catharus ustulatus*).

Song type matching, in which a singer replies to a conspecific song type by singing the same song type, is another vocal behaviour often labeled as being an aggressive signal. Some research, mainly in song sparrows, suggests that matching represents an intermediate signal in a hierarchical system that begins with vocal interaction and potentially escalates to approach, soft song, and physical attack (Searcy and Beecher, 2009; Ackay et al., 2013). Hermit thrush males do not appear to share song types extensively, and therefore cannot match song types. However, because they have distinct low and high frequency song types, they may instead frequency match, as occurs in Harris' sparrow. Harris' sparrow males have similar high and low categories of song type frequencies and sing with similar syntax to hermit thrush (Shackleton et al., 1991).

The results of this study indicate that hermit thrush males do not frequency match like Harris' sparrows but rather do the opposite, actively avoid it. When the eastern playback recording broadcast a high or low frequency song type, the subject followed with the opposite song type (i.e., responded to a high song type with a low song type, or vice versa) more frequently than would be expected by chance. Kroosdma (2009), analyzing one individual hermit thrush's vocal behaviour, also observed avoidance of song matching. Especially considering the lack of correlation between rate of matching and magnitude of physical response to playback, it appears that frequency matching does not play an aggressive role here, as it does in song sparrows. It is possible that avoidance of matching minimizes sound interference and increases transmission efficiency, or relates to mate attraction. Interactive playback experiments, in which the vocal and physical responses of territorial males to playback that frequency matches their own songs to varying degrees, may provide insight into whether matching avoidance plays any role in vocal interactions between males.

6.4.4 Conclusions

In agreement with my hypothesis, eastern hermit thrush males exhibited a stronger territorial response to local songs from the same subspecies compared to songs from a distant, western subspecies. This finding indicates that males can discriminate between local and foreign songs, and suggests that they are capable of discriminating on the basis of subspecies and/or subspecies group. In addition, this study revealed that, during aggressive vocal interactions, territorial males employ unique versions of their songs that lack introductory notes and have not been observed in other contexts. Lastly, in terms of other vocal interactions, the findings revealed that hermit thrush males actively avoid overlapping conspecifics and also avoid frequently matching by responding to playback with songs occupying different frequency bandwidths.

With respect to the differential responses to local versus foreign songs, future research should elaborate on these findings using playback stimuli that differ to different degrees (different subspecies, local versus distant within subspecies, neighbour versus stranger) in order to more fully elucidate how conspecific recognition varies within distance and song differences. In terms of understanding the current evolutionary trajectory of the species, and the role of song variation therein, playback within the hybrid zone between eastern and western lineages may provide valuable insights. Further playback studies will also reveal if hermit thrush males of other subspecies are similar to those in this study with respect to avoidance of overlapping and matching, as well as the use of abridged versions of broadcast songs in aggressive contexts. Another important avenue for future research is to examine female song preferences, including comparison of conspecific recognition by females to that of males. Research on how females assess males based on song structure and/or singing behaviour may also help us to better understand the functional roles of hermit thrush song components.

CHAPTER 7: GENERAL DISCUSSION

Among songbirds, perception of vocalizations plays a vitally important role in conspecific recognition: both males and females often identify individuals as belonging to the same or a different species on the basis of song structure (Catchpole and Slater, 2008). Further, birds may use acoustic features of vocalizations, and of song in particular, to discriminate between individuals and assess quality. Recognition therefore has important consequences: for males, conspecific recognition helps to categorize the level of threat posed by a nearby individual; in the case of females, it is a necessary precursor to assessing potential mates and making reproductive decisions on that basis (Catchpole and Slater, 2008). As with most aspects of song production (e.g., song structure, repertoire size, and syntax), the song components that signal an individual as being conspecific vary greatly across songbird species. Thus, not just production of acoustic signals (e.g., song) but also perception and relative importance of particular acoustic features vary greatly among songbirds.

The research described in this thesis explored aspects of song perception, and specifically conspecific recognition, in two songbird species: the black-capped chickadee and the hermit thrush. These species differ substantially in a number of respects, including basic song structure and syntax, as well as the amount that is known about each species' singing behaviour. Reflecting these differences, the research described herein examines conspecific recognition from somewhat different approaches for these species. The results provide valuable information on vocal communication in each of the species, adding to our broader understanding of how songbirds use their songs and process those of conspecific individuals.

The first two studies described in this thesis (Chapters 2 and 3) were conducted using the well-described and studied song of the black-capped chickadee. A common songbird with a structurally simple song (*fee bee*), the chickadee has been used in numerous studies related to song production and perception. The *fee bee* song lends itself well to manipulation for studies of conspecific recognition for two reasons. First, it is a simple, two-note (*fee* and *bee*) song. Second, even when sung at different absolute pitches, the song retains two highly consistent relative pitch ratios: the ratio from the start to the end of the *fee* note (the *glissando*) and the frequency ratio between the end of the *fee* note and the start of the *bee* note (the inter-note interval). Therefore, testing the importance of the relative ratios to conspecific recognition, as well as the ability of birds to perceive those differences, is a matter of altering those mathematical ratios within the song; such elegant manipulations would be much more difficult in a more complex song.

Behavioural studies indicate that conservation of these ratios is critical for recognition: females respond less strongly to songs with either of those ratios altered, indicating that they attend to both of the pitch ratio rules described above (Ratcliffe and Otter, 1996). In contrast, males respond less strongly to songs with the *glissando* removed but not to songs with the inter-note interval altered, suggesting that they attend to only one of the two rules (Shackleton et al., 1992). In the first two studies of this thesis (Chapters 2 and 3), I extended this behavioural research by first looking at whether altering the ratios affected neural response in regions important for perception and then examining chickadees' abilities to discriminate among songs with and without such alterations in a controlled setting.

Although the results of these two studies indicate that birds can discriminate and attend to songs based on the presence of pitch ratios, this was not reflected in neural responses. In response to playback of songs with species-typical pitch ratios and songs with those ratios altered, there were no differences in neural activity within auditory regions (caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM)) that have previously been associated with discrimination of conspecific versus heterospecific vocalizations (Chew et al., 1996). Aligning with recent evidence that structural differences that are sufficiently small are not discriminated in NCM and CMM (Avey et al., 2014), these results suggest that the subtle discriminations involved in detecting the presence or absence of species-typical pitch ratios may take place at higher levels within the auditory system, such as HVC. The relationship between the magnitude of differences between vocalizations (and, therefore, the complexity of the discrimination) and the level within the auditory system at which discrimination takes place remains unclear and should be a focus of future research, both in chickadees and in other songbird species.

Vocal behavioural responses to playback (Chapter 2) and performance during operant discrimination training (Chapter 3) paralleled each other with respect to assessment of normal and altered songs. Both males and females vocalized more in response to songs with a species-typical inter-note pitch ratio than to songs with that ratio reduced to 1.00, indicating that chickadees in this study attended to the inter-note interval but not to the *glissando*. Results of my operant study mirrored these results, as birds easily learned to discriminate songs with the inter-note interval from songs without it; in contrast, they had difficulty learning to discriminate between songs with and without the *glissando*. Thus, chickadees in this study not only did not attend to the *glissando* but also had difficulty discriminating its presence versus absence.

This contrasted with previous behavioural work suggesting that females attend to both pitch ratios, while males attend to only the *glissando.* Differences between previous behavioural studies and the present results may relate in part to methodological differences. For example, Shackleton et al. (1992) measured territorial responses in the wild; it is possible that males in this study, captive and hearing stimuli presented in a completely different environment, did not process the songs as a potential territorial threat in the same way they would in nature. Although the previous behavioural work with female chickadees was conducted in a lab environment, like this study, there were nonetheless methodological differences: Ratcliffe and Otter (1996) measured a different element of female response (solicitation displays) and used estradiol implantation.

Together, the results of these studies highlight the need for further research on which aspects of the *fee bee* song are of biological importance to male and female chickadees. As suggested above, one difficulty in evaluating the existing evidence is that the relevant studies have taken place in different experimental contexts, with data on male responses collected in the field and data on female responses collected in the lab. Further, those studies have measured different types of responses. In order to get a comprehensive understanding, there is a need for studies that evaluate both male and female responses to normal and altered songs in a natural environment (and within the same experiment). The findings of such studies will provide the necessary foundation for further work examining the neural underpinnings of how male and female chickadees recognize and assess conspecific songs.

In the third, fourth, and fifth studies, I examined conspecific recognition in the hermit thrush, a songbird that differs from the black-capped chickadee not only in basic aspects of singing behaviour (e.g., repertoire size, structural complexity of songs) but also with respect to what we know about its songs and how they are used. To address the relative lack of information regarding hermit thrush song, I first performed a detailed bioacoustics analysis of hermit thrush song, examining song structure, repertoire size and composition, and song syntax (Chapter 4). This basic information not only laid the groundwork for Chapters 5 and 6 in this thesis but also provides a foundation for studies of various aspects of hermit thrush song by other researchers. As a common and widespread songbird with a moderate repertoire size and interesting song syntax, the hermit thrush may, like the black-capped chickadee, have great utility as a model species for studying various aspects of birdsong.

Researchers have documented extensive variation in genetics (Kerr et al., 2007; Topp et al., 2013; Alvarado et al., 2014), morphology (Aldrich, 1968; Alvarado et al., 2014), and migratory patterns (Dellinger et al., 2012; Alvarado et al., 2014) across hermit thrush subspecies and subspecies groups. However, comparatively little is known about geographic variation in hermit thrush song structure, despite song's potentially important role in evolutionary processes including speciation (Slabbekoorn and Smith, 2002a). Addressing this gap, Chapter 5 documents an array of song structure differences both within and between major subspecies groups. For example, the introductory note, which may serve an alerting function to capture the attention of distant potential mates, occupied dramatically different frequency spectrums within eastern and western lineages.

Understanding geographic song differences is especially important in light of recent studies documenting a genetic divergence between eastern and western lineages of hermit thrush that is sufficiently large to suggest division into two separate species (Kerr et al., 2007; Alvarado et al., 2014). Song plays a central role in reproduction: before any mate choice and mating can take place, females must first, on the basis of song, recognize a male as a conspecific. Thus, divergence in song structure can influence conspecific recognition, in turn affecting mating patterns and contributing to reproductive isolation and speciation, as has been documented in other songbird species (e.g., Irwin et al., 2001).

Given the potential of song differences to contribute to the kinds of evolutionary processes that appear to be underway in hermit thrush (Kerr et al., 2007; Alvarado et al., 2014), it is important to go beyond an understanding of geographic patterns in song structure and explore how such differences influence species recognition. The playback study described in Chapter 6 provides an important first step in that direction by comparing the territorial responses of males from an eastern subspecies to playback of songs from the same local population and songs from a distant subspecies belonging to the western lineage. Males in this study exhibited a stronger and more aggressive territorial response to local songs of familiar structure compared to songs that were from a distant subspecies and differed substantially in structure. Specifically, the eastern subjects responded to eastern playback more quickly, spent more time close to the sound source, and performed more flights. These findings provide valuable context to our emerging view of hermit thrush genetic divergence and possible speciation, showing that birds exhibit stronger song-based recognition of individuals from their own lineage than those of the other lineage.

Further work will be required in order to determine the degree to which the response differences observed reflect distinctions between lineages, between local versus foreign, or some combination of both. In addition, similar studies using subjects from the western lineage are necessary in order to determine if the discrimination observed here is reflected elsewhere across the hermit thrush breeding range; asymmetric discrimination, documented in some other songbirds, can have distinct influences upon reproductive patterns and evolutionary trajectories (e.g., Colbeck et al., 2010; Dingle et al., 2010). Lastly, and perhaps of greatest relevance, future research should explore female responses to local versus foreign songs; this will provide more direct information on how song divergence influences conspecific recognition in the context of mate choice.

Future work in these and other songbird species should strive to understand how song transmits information about species identity, as well as more specific information such as individual identity and male quality. While the pitch ratios in black-capped chickadee songs have been associated with conspecific recognition by previous behavioural studies (e.g., Shackleton et al., 1992), one of those ratios, the inter-note pitch interval, may also convey information about dominance and be used by females in mate choice (Christie et al., 2004b). In hermit thrush, it remains unclear which song attributes are necessary for conspecific recognition, although the novel vocalizations observed in response to aggressive playback (Chapter 6) suggest that the introductory note may serve a long-range function, possibly related to species recognition.

Another objective for future studies should be to further clarify the roles, together and separately, of absolute and relative pitch processing. While songbirds are, overall, more adept at absolute that relative pitch processing tasks, there is evidence that they can use both strategies in concert (MacDougall-Shackleton and Hulse, 1996). In particular, there would be value in examining the use of absolute and relative processing strategies in species where relative pitch is important to song recognition (e.g., black-capped chickadees, veeries) because such species employ relative pitch processing to a greater extent than other songbirds. According to recent research, the notes within the post-introductory portion of hermit thrush song follow pitch ratio rules similar to those found in human music (Doolittle et al., 2014). Thus, hermit thrush may be another species that relies on relative pitch processing in order to extract information of biological significance.

In conclusion, the research in this thesis examined conspecific recognition in ways suited to the singing behaviour of each of the species studied. For black-capped chickadees, the results provided further information on the song components needed for recognition and the abilities of male and female chickadees to detect those components. The studies with the hermit thrush provided vital information on singing behaviour; they also explored geographic song variation and its potential influence upon song perception, placing that information in the context of recent work regarding the species' evolutionary trajectory.

The two songbirds species studied here differ considerably not just in terms of singing behaviour, but also in other respects including genetic make-up. In contrast to the deep genetic divergence observed in hermit thrush, the genetics of black-capped chickadees are remarkably homogeneous across their breeding range (Foote et al., 2010a). Likewise, black-capped chickadee song varies little across its range (Kroodsma et al., 1999). Nonetheless, even if song production differs little among black-capped chickadee populations, it is possible that aspects of song perception vary. For example, the relative amplitude of the *fee bee* song two notes appear to signal male dominance in a western black-capped chickadee population, but not in another, eastern one (Hahn et al., 2013a). In black-capped chickadees and other species, future research should strive to understand song production and perception in concert, allowing for a more complete picture of how song carries meaning to both male and female songbirds and informs behavioural decisions.

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