

Running head: HVC AND SONG PRODUCTION

THE ROLE OF HVC IN COORDINATING ANATOMICALLY ASYMMETRIC SOUND
SOURCES WITH A BILATERALLY CONTROLLED RESPIRATORY SYSTEM

by

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Submitted to the Assigned Dissertation Committee of the
College of Science and Engineering
Texas Christian University
In partial fulfillment of requirements
for the degree of

Doctor of Philosophy

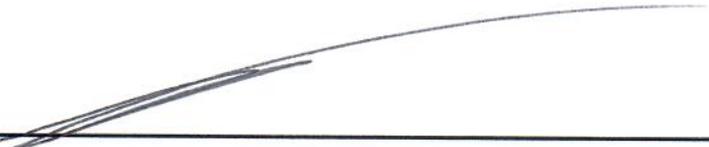
August 2017

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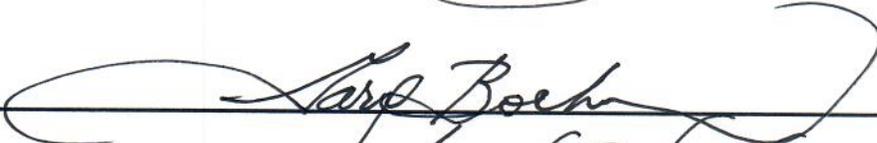
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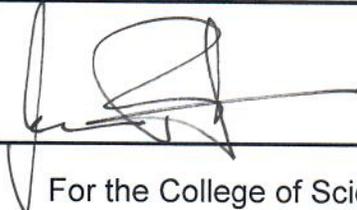
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For the College of Science and Engineering

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Preface

Chapters in this dissertation have been organized in preparation for publication

This document is dedicated to my grandparents:

Mario and Lily Mancenido

Aanhin pa ang damo kung patay na ang kabayo?

Deepest thanks to my family for years of support.

I would like to express my gratitude to Dr. Brenton Cooper, for excellent mentorship and efforts to turn myself into a decent scientist and team player.

Special thanks to my committee: Dr. Tim Barth, Dr. Gary Boehm, Dr. Michael Chumley, and Dr. Ken Leising for taking the time and effort to help me succeed.

Many thanks to Dr. Chumley for sharing his expertise and lab equipment.

Special thanks to Dr. Samuel Sober and Dr. Kyle Srivastava for assistance with dTB surgeries, Dr. Franz Goller, Dr. Kazuo Okanoya and Dr. Tobias Riede for insight shared with the lab.

Thank you to my lab mates (in chronological order):

Kristen Secora

Boah Chung

Jen Peterson

Emily Spradley

Avery Aston

Kara Marie Lane

James Taylor

Austin Lemke

Marshall Watts

Bottomless thanks to Lindy Bledsue for helping us keep our animals safe and well over many, many years.

Additional thanks to former and current graduate students for years of feedback during Biopsych: Dr. Marielle Weintraub, Dr. Dinko Kranjac, Dr. Tracy Lepper, Dr. Amanda Glueck, Dr. Micah Eimerbrink, Josh Wolf, Jordon White, and Bailey Devine

Extra special thanks to Dr. Jen Peterson and Dr. Marielle Weintraub for friendship and support starting 2 BGS (Before Graduate School).

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Chapter 1: Songbirds as animal models for vocal development

Fundamentals of birdsong

Birdsong is a behavior with a rich array of vocalizations. Depending on the species, a songbird can sing one song or possess a repertoire of hundreds of song types. For every species, acoustic and/or temporal characteristics make their song unique and recognizable. Some species also have regional dialects and subtle variations can identify a singer's geographic origin. This is most evident in white-crowned sparrow (*Zonotrichia leucophrys*) song where all males in a local population will sing the same song, albeit with individual variation (Marler & Tamura, 1962). Furthermore, studies with zebra finches (*Taeniopygia guttata*) show siblings who learn their song from the same tutor will develop songs with varying degrees of accuracy (Böhner, 1983; Tchernikovsky, Lints, Mitra & Nottebohm, 1999). As a result, their adult song becomes as unique as a human fingerprint.

Despite the diversity in singing behavior, there are many commonalities in acoustic and temporal characteristics. Song follows a temporal hierarchy and song behavior is produced in a stereotyped and repetitive manner. It is typically generated in either a sexual or territorial context, and therefore is shaped by both inter- and intra-sexual selection. The primary components of song are notes, syllables, trills, and motifs. A note is a single sound and multiple notes can be joined together to form a syllable, the smallest cohesive unit of song (Baker, 2001). A syllable, whether produced by continuous or separated notes, is preceded and followed by 10 ms of silence. Syllables can be produced with a constant frequency or with rapid frequency modulation. Trills

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are successive repetitions of the same syllable. Syllables can be combined in a fixed sequence to form a motif, which is then repeated multiple times to form a song bout. As song behavior is highly stereotyped, acoustic characteristics are often employed as a means of quantifying the phonological structure of the syllables. This is useful for descriptive purposes as well as providing a method for comparing songs across individuals and species.

Parallels between human and oscine vocal acquisition

Human infants are exposed to their native language beginning with auditory experience in the womb. Near term fetuses are able to discriminate their mother's voice from a stranger's and can detect a speaker's voice changing from male to female or vice-versa (Kisilevsky et al., 2003; Lecanuet, Granier-Deferre, Jacquet, Capponi, & Ledru, 1993). Even as newborns, they are able to discriminate like consonants such as /ba/ or /da/ and they are sensitive to rhythm and intonation that distinguishes lexical words from grammatical words (Shi, Morgan, & Allopenna, 1998a). Their constant exposure to parent speech enables them to later discriminate vowels and other grammatical cues important to their language (Hirsch-Pasek et al., 1987; Jusczyk, 1997; Kuhl, 1979). The songbird model of human vocal learning is used primarily because song, like human speech, is a learned behavior that requires exposure to a conspecific tutor. Juveniles use the conspecific song as a memorized model and refine their own vocalizations to approximate the remembered song. This auditory-dependent song learning has been demonstrated by rearing birds in isolation, and therefore without exposure to conspecific song, and by deafening at different time points during

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development (Immelman, 1969; Konishi, 1965). Zebra finches and Bengalese finches reared by hand and isolated from other males sing simple, uniform, and unadorned “isolate” songs that lack any typical characteristics of male vocalizations (Immelman, 1969; Mendez, et al., 2009; Felga, et al., 2016).

Further, deafening studies confirm that the ability to hear one’s own vocalizations is critical for normal vocal acquisition. Children that lose their hearing before puberty experience rapid vocal deterioration as compared to the mild speech deficits of late-deafened adults (Cowie & Douglas-Cowie, 1992; Plant & Hammarberg, 1983; Waldstein 1989). White-crowned sparrows do not rehearse their song until weeks after exposure to the tutor (Figure 1B). Therefore, they were ideal for studying the role of auditory feedback in vocal development because vocal production does not overlap with auditory memory formation. Juveniles were either raised in isolation or with exposure to conspecifics, and then they were deafened during the sensorimotor phase of song learning. Both groups produced an abnormal song lacking in phonological or sequential organization. These songs lacked similarity to conspecific or isolate songs (Konishi, 1965). Further studies with deafening juvenile chaffinches (*Fringilla coelebs*) at various stages in the sensorimotor phase show that removing auditory feedback arrests a juvenile’s capacity for generating complex song units characteristic of the normally reared adult song (Konishi & Nottebohm, 1969).

There is a sensitive period for juvenile songbirds to acquire normal song that is similar to a child’s acquisition of spoken language (Doupe & Kuhl, 1999). The timeline of song acquisition varies from species to species, but all songbirds go through three critical phases (Figure 1A,B). During the initial sensory phase, a juvenile listens to the

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adult's song and uses it to form a memorized template of the basic song structure. This is then followed by the sensorimotor phase wherein the young bird begins to produce subsong. Subsong is composed of unstable, quiet, low-frequency sounds that are monotone and rather indistinguishable from each other (Marler, 1970). As song-related vocalizations become structurally complex and begin to resemble adult song syllables, the juvenile is considered to be in its plastic song phase. Juveniles then rehearse their plastic song until it matures into adult species-typical sequences (Brainard & Doupe, 2000; Immelman, 1969; Marler, 1970). Similarly, infants produce nonspeech sounds for the initial three months of life. These sounds mature into strings of consonant-vowel pairs (e.g. "babababa") at approximately 7 months. Their first word emerges when the child is one year old (Figure 1C; Darley & Winitz, 1961).

Infants and juveniles show general auditory sensitivity during the early stages of development. Until approximately 6 months of age, human infants are able to discriminate phonetic units of both native and nonnative languages. As they grow older, infants become attuned to phonetic differences that are important to their native language and lose discriminative ability with non-native sounds (Doupe & Kuhl, 1999; Werker & Tees, 1999). Young oscines are similarly able to copy heterospecific song in the absence of conspecific tutors. Studies show that cross-fostered zebra and Bengalese finches readily imitate the songs of their heterospecific foster tutor (Immelman, 1969). Additionally, male brown-headed cowbirds (*Molothrus ater*) that spent the first year of their life with canaries (*Serinus canaria*) learned the canary song repertoire (West, King, & Freeberg, 1996).

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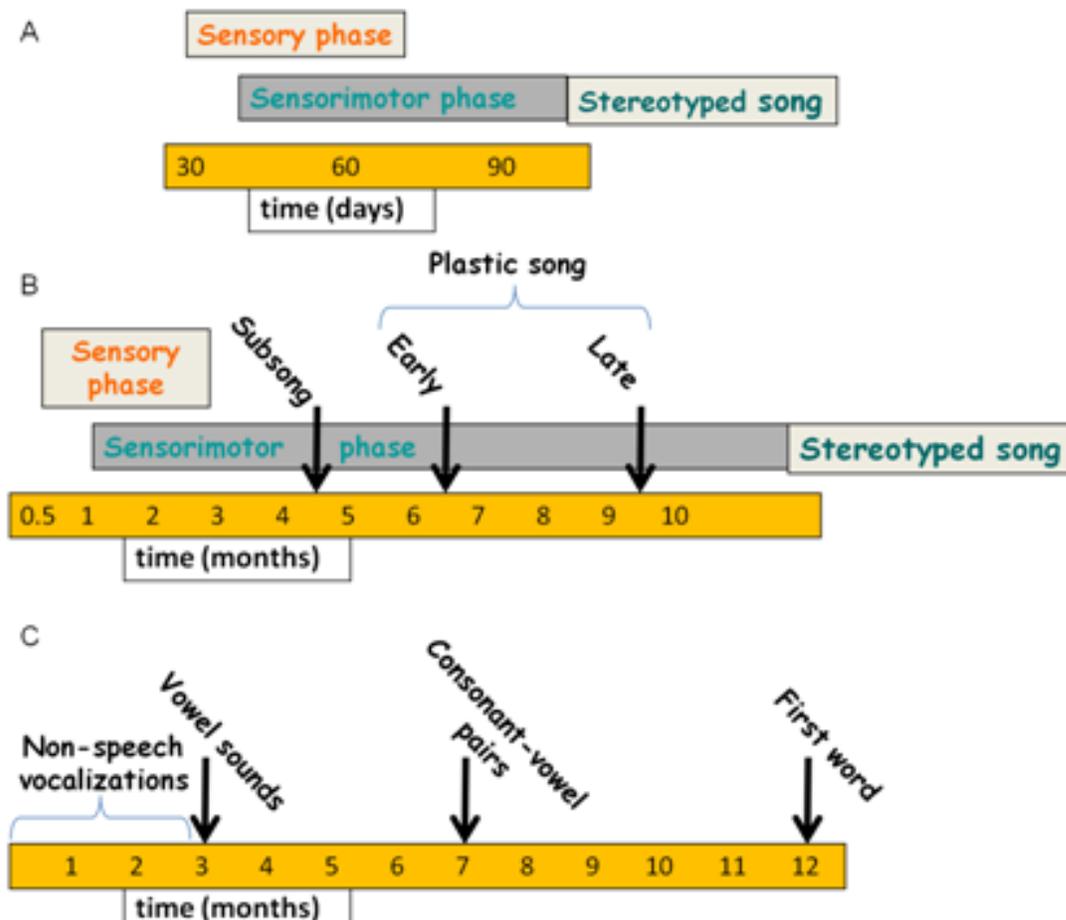


Figure 1. Critical periods in vocal development. A) Zebra finches have a great deal of overlap between sensory and practice phases and acquire their adult song by 90 days post-hatch. B) White-crowned sparrows acquire the song template by 7 weeks post-hatch but do not begin rehearsal for another two months and their song can be modified until 1 year post-hatch. C) Human infants begin producing speech sounds at 3 months of age. They learn to produce consonant-vowel combinations at 7 months and at 12 months, they being producing simple words.

Neural pathways of song production

The mammalian neocortex is organized into six layers of neurons, with each layer having a unique pattern of neuron types and organization. The avian analogue of the neocortex is segregated into clusters of neurons, or nuclei, and each nucleus has its own unique anatomical and electrophysiological properties (Nottebohm et al., 1990;

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Mooney, 1992). A discrete set of interconnected nuclei are implicated in song learning and production. These networks of song-related nuclei are typically larger in males than in females (Nottebohm & Arnold, 1976). Further, song nuclei are not found in closely related suboscines, or other species that do not display vocal learning (Gahr, Guttinger, & Kroodsma, 1993).

Of particular interest to songbird neuroscientists are two interconnected forebrain pathways important for the development and maintenance of song behavior. These are termed the direct “posterior vocal motor pathway” and the indirect “anterior forebrain pathway” (Figure 5). Both pathways are innervated by the premotor nucleus HVC (letters used as proper name) and terminate in RA (robust nucleus of the arcopallium), which is the motor nucleus of the song system (Nottebohm, Stokes, & Leonard, 1976; Nottebohm, Kelley, & Paton, 1982; Gurney, 1981; Bottjer, Halsema, Brown, & Miesner, 1989).

The direct pathway links the premotor song nucleus, HVC, to RA. Neural recordings from both HVC and RA suggest that motor commands likely flow from HVC to RA and then to the syrinx in a hierarchical manner (Hahnloser, Kozhevnikov, & Fee, 2002). RA’s other input emerges from the forebrain nucleus, lateral magnocellular nucleus of the anterior nidopallium (LMAN), the output nucleus of the anterior forebrain pathway, which is critical for juvenile song learning and song modification in adulthood (Brainard & Doupe, 2001; Bottjer, Miesner, & Arnold, 1984; Kobayashi, Uno, & Okanoya, 2001; Scharff & Nottebohm, 1991; Sohrabji, Nordeen, & Nordeen, 1990; Williams & Mehta, 1999). RA encodes motor commands essential for the stable formation of song units. Ventral RA outputs provide fine-motor control of song via the

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tracheosyringeal portion of the hypoglossal nucleus. This nucleus provides unilateral neuromuscular innervation of the syrinx, or avian vocal organ (Vicario, 1991b; Wild, 1997). Dorsal RA projects to brainstem nuclei that control expiration (nucleus retroambigualis, RA_m) and inspiration (nucleus paraambigualis, PA_m).

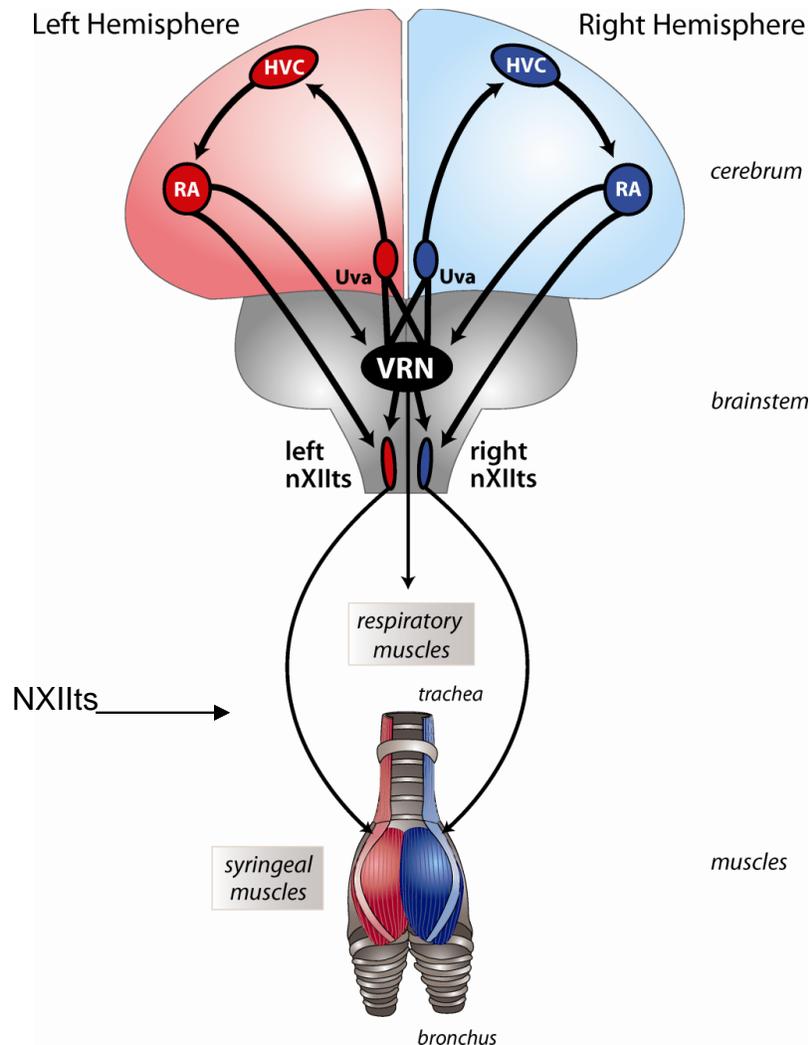


Figure 2. Unilateral neural control of the oscine sound generator. The final motor commands from the left (red) and right (blue) sides of the brain provide neuromuscular innervation of the ipsilateral side of the syrinx for manipulating the vocal tissue. The song circuit provides neuromuscular commands to the respiratory system via nuclei RA_m and PA_m, part of the ventral respiratory network (VRN) Abbreviations: HVC (used as a proper name), RA = robust nucleus of the arcopallium; nXIIts = tracheosyringeal portion of the hypoglossal nucleus; NXIIts = tracheosyringeal portion of the hypoglossal nerve. © Mark Schmidt, 2008.

Syringeal Lateralization and Implications for Songbird Neural Control

The syrinx is a bifurcated vocal organ attached to the caudal end of the trachea where it meets two bronchi. In songbirds, there are two sets of vibratory tissue within each bronchus, each controlled independently by the ipsilateral hemisphere (Figure 2). The external facet is composed of pairs of syringeal muscles, all innervated by the tracheosyringeal portion of the hypoglossal nerve (NXIIIts; Wild 1993a, 1997; Figure 3). Syringeal muscles coordinate the motor gestures that produce each syllable. Electromyogram (EMG) recordings of individual syringeal muscles in brown thrashers (*Toxostoma rufum*) show that the dorsal syringeal muscles (*m. tracheobronchialis dorsalis*) contract to move the avian vocal tissue, the medial and lateral labia, into a partially obstructive, phonatory position or to close the airway completely (Goller & Suthers, 1995b, 1996b; Figure 4). Preventing airflow through one side of the syrinx silences one of the two sound sources within the vocal organ. Ventral syringeal muscles (*m. ventralis syringealis*, vS) participate in the finer motor gestures that control the fundamental frequency of syllables. In brown thrashers, EMG activity in ventral muscles is correlated with the fundamental frequency. vS activity is also correlated with frequency modulation (Goller & Suthers, 1996b). In zebra finches, vS activity correlates with both frequency and vocal tissue adduction during inspiration (Goller & Cooper, 2004;2008).

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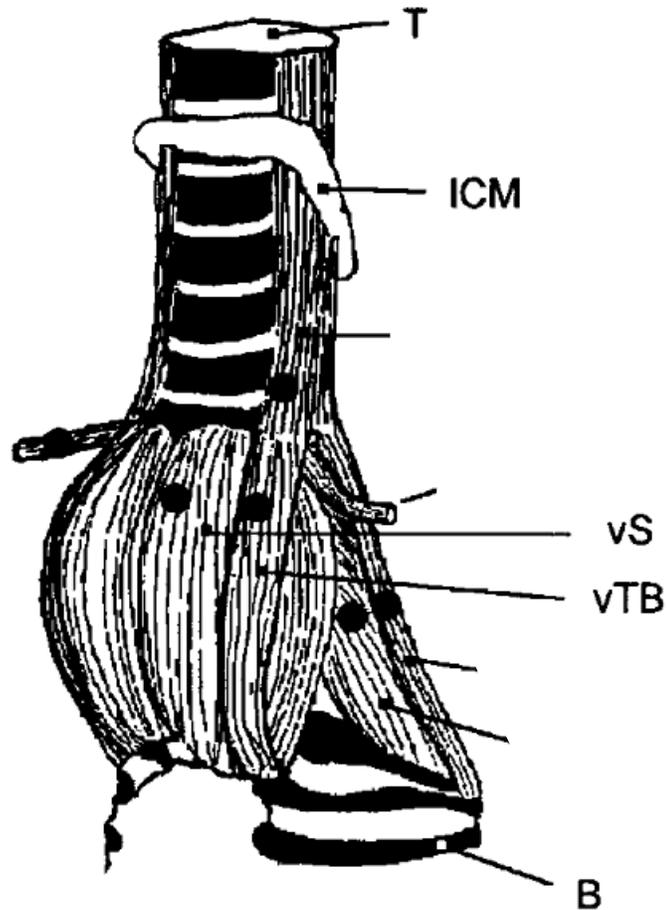


Figure 3. Ventrolateral external view of the brown thrasher syrinx. *m. tracheobronchialis dorsalis* (vTB) contract to either close the airway or move vocal tissue into phonatory position. *m. syringealis ventralis* (vS) may provide fine-control of vocal tissue tension to produce a range of fundamental frequencies. All syringeal muscles receive motor commands via the tracheosyringeal portion of the hypoglossal nerve, NXIIIts. Abbreviations: T = trachea, ICM = membrane of the interclavicular air sac, B = bronchus . © Rod Suthers, 1997.

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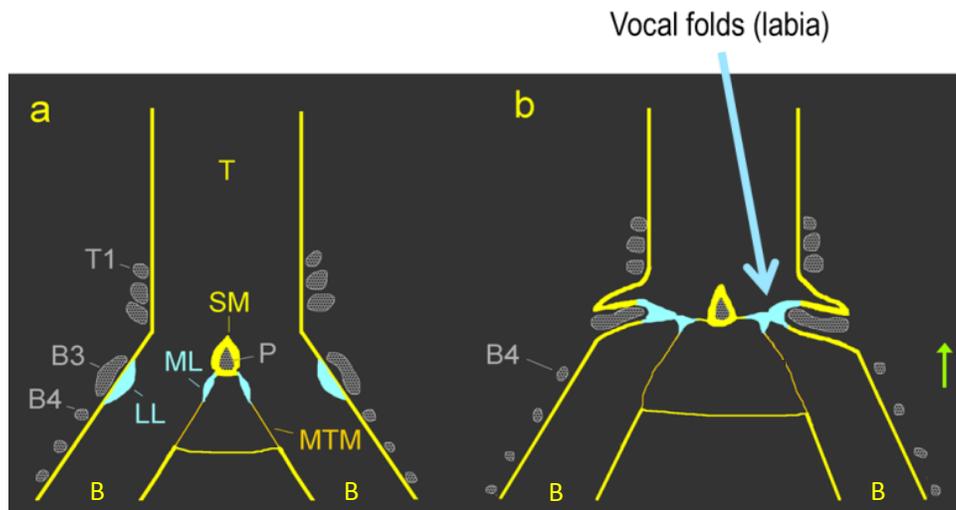


Figure 4. Cross sectional diagram of the songbird syrinx. Note the position of the vocal tissue (light blue) in the A) resting, and B) phonatory position. Air leaves the body via the bronchial tubes and trachea (green arrow). During phonation, air is forced between semi-obstructive vocal tissue, causing the vocal tissue to vibrate and produce sound. T = trachea, B = bronchial tube.

The oscine syrinx is capable of two-voice sound production because of the vibratory tissue, the medial and lateral labia, present in the caudal area of both bronchi. To generate tonal notes, a songbird can either generate sound using the left or the right sound source (with the nonphonatory side typically closed) or simultaneously vibrate both sound sources at the same frequency. Alternatively, each sound source can vibrate at harmonically related frequencies to generate an overtone, an integer multiple of the two fundamentals. Both sound sources can also simultaneously generate harmonically-unrelated frequencies to create sounds with a “noisy” timbre.

Unlike mammals, birds do not have a corpus callosum. This raises the interesting question of how activity between the two hemispheres is coordinated in the absence of commissural projections. Does each hemisphere operate independently? One possible scenario is that one hemisphere is in complete control of song production. Just as

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humans are lateralized in the speech and language domains, initial work suggested that songbirds seemed to exhibit hemispheric dominance. Species such as Waterslager canaries (*Serinus canaria domesticus*, Nottebohm & Nottebohm, 1976), chaffinches (Nottebohm, 1970, 1971a, 1972), Java sparrows (*Padda oryzivora*, Seller, 1979), white-throated sparrows (*Zonotrichia albicollis*, Lemon, 1973), and white-crowned sparrows (Nottebohm et al., 1976) show left-hemispheric dominance for song production.

Abolishing left hemisphere motor signals by removing the left NXIIIts nerve destroys syllable phonology and makes their song virtually unrecognizable. In Waterslager canaries and chaffinches (Nottebohm, 1971b; Nottebohm & Nottebohm, 1978), the lateralization of vocal behavior is experience-dependent. If canaries experienced left NXIIIts resection shortly after fledging from the nest, they were able to compensate and acquire the species-typical song. Zebra finches have right-hemisphere dominance as right side syringeal denervation results in an immediate loss of acoustic structure and syllables become largely unidentifiable (Price, 1977; Williams, Crane, Esposito, & Nottebohm, 1992).

A second possible scenario is that frequency control is lateralized and neural control of song is divided between the two hemispheres. The zebra finch uses the right side of the syrinx to produce higher frequency notes and syllables compared to the left sound source (Goller & Cooper, 2004). This division of syllable frequency control has also been observed in gray catbirds (*Dumetella carolinensis*, Suthers, 1990), brown thrashers (Goller & Suthers, 1996b) and brown-headed cowbirds (Allan & Suthers, 1994). While some overlap in frequency production occurred, each sound source was responsible for a selective portion of the frequency range, with the right syringeal sound

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source typically controlling higher frequency sound production. These findings are supported by recent anatomical research into the vocal tissue of several species of songbirds that show the morphology and composition of the vocal tissues largely determines fundamental frequency (Riede & Goller, 2014). Further, EMG recordings of dorsal syringeal muscles show that both sound sources receive continuous neuromuscular commands while birds are singing. For example, when a bird sings with only one sound source, there is adduction of the vocal tissue into a phonatory state on that side, and silencing of the nonphonating sound generator requires active muscular control to close the airway (Goller & Suthers, 1995a).

Unilateral RA lesions in adult zebra finches, whose song circuit is fully mature, show complete disruption of song after RA ablation in the left or right hemisphere. On the other hand, juveniles with unilateral RA lesions learn to sing a normal adult song, even in the absence of a normally functioning left or right RA (Ashmore, Bourjaily & Schmidt, 2008). This suggests that in intact adult birds, the two hemispheres do not operate in isolation and must be communicating to coordinate the two sound sources. The only known projections between the left and right sides of the brain occur in midbrain and brainstem, particularly in nuclei controlling respiration and calling behavior (Schmidt & Ashmore, 2008; Vicario & Simpson, 1995). As there are no known projections between telencephalic nuclei, it is most likely that the two hemispheres are using bottom up projections to synchronize the two sound sources (Schmidt, 2003)

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Syringeal Lateralization of the Bengalese Finch

Lateralized song production in Bengalese finches (*Lonchura striata domestica*) is drastically different from previously investigated species in two ways. First, higher frequencies of the song are produced with the left sound generator, which is the opposite of previously studied species such as the zebra finch (Goller & Cooper, 2004), northern cardinal (*Cardinalis cardinalis*; Suthers & Goller, 1996), white-crowned sparrow (Nottebohm & Nottebohm, 1976), mimic thrushes (Suthers et al., 1994), brown thrashers (Goller & Suthers, 1996) and brown-headed cowbirds (Allan & Suthers, 1994). Second, there is very little overlap between the contributions of the left and right sound generators. This, again, is quite unusual. While it is well-known that songbirds take advantage of their dual sound generator by dividing their vocal range between the two sound generators, sounds produced by each side are similar in other acoustic characteristics. Bengalese finches, on the other hand, contribute higher frequency tonal components with the left sound source and noisy components with the right sound source. Denervation of the left syringeal muscles caused the birds to sing songs that were no longer similar to their intact songs. They now sang with a lower amplitude, lower fundamental and peak frequency, and with increased entropy compared to the songs produced prior to left NXII resection (Figure 5, Secora et al., 2012). In sharp contrast, after right nerve resection, syllables were identifiable and higher frequency components were undisturbed. “Noisy” acoustic sounds that typically appeared in two-voice stacks and at the first 5-7 ms of a syllable were lost following right NXII resection (Figure 6). These data suggested a strong lateralization of acoustic production between the two syringeal sound sources in Bengalese finches.

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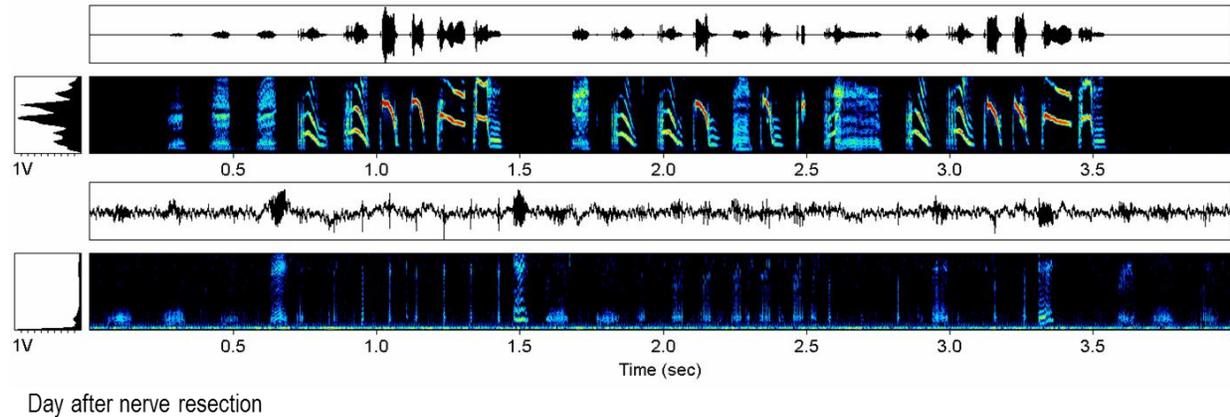


Figure 5. Acoustic effects of L-NXIIIs resection. Spectrogram of male Bengalese finch song prior to and after left NXIIIs resection. Sound is depicted along time (x-axis) and frequency (y-axis) and color coded for amplitude. Louder (red-coded), higher frequency components are no longer present after surgery.

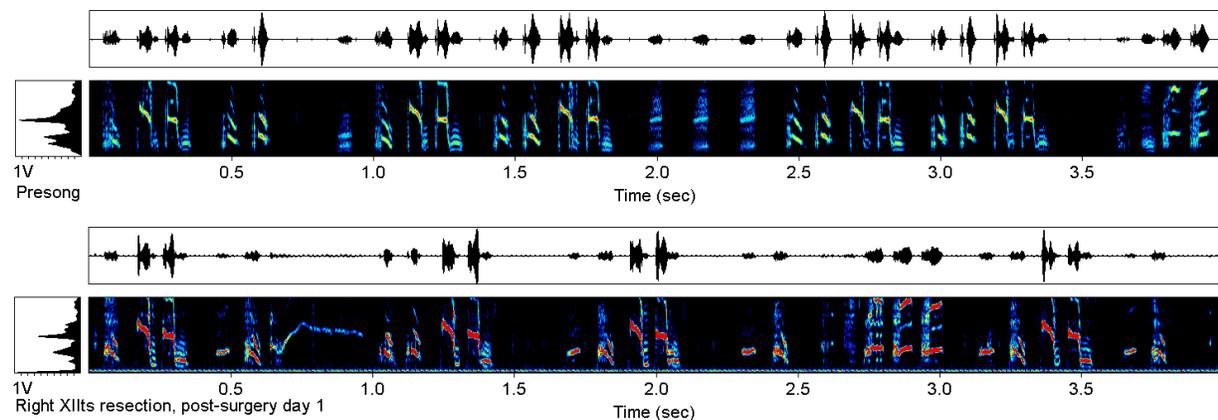


Figure 6. Acoustic effects of R-NXIIIs resection. Spectrogram of male Bengalese finch song prior to and after right NXIIIs resection. Note that syllables remain largely unchanged.

The contribution of each syringeal sound generator was further assessed through unilateral devocalization. By surgically extracting the third bronchial cartilage, one set of lateral labia was removed. The subsequent healing process sealed the airway and left birds with only one potential sound generator. After left devocalization, birds sang with only the right sound source and produced song that consisted of high-entropy “clicks”

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that occur at syllable onsets. The retention of “clicks” but loss of higher frequency notes demonstrates that the right side of the syrinx produces high entropy sounds. Further, no notes or syllables with a fundamental frequency higher than 2.2 kHz were observed following loss of the left sound source suggesting that the left sound source produces notes in this frequency range. The opposite pattern was observed when the right sound source was devocalized, and birds sang with the left sound source. The Bengalese finches continued to sing higher frequency notes (above 2.2 kHz) and no longer produced “clicks” at the onset of syllables (Secora et al., 2012).

Replicating the devocalization procedure with females, who do not learn their vocalizations, showed the same pattern of acoustic lateralization and suggests that the lateralization is not acquired through social learning (Urbano, 2013). Evaluation of syringeal vocal tissue in both males and females supports the notion that the lateralization is due to the asymmetric structure of the vocal tissues in the two sound sources (Urbano, 2013). For the vocal tissues, the relationship between tissue length and tissue stress, the viscoelastic property, is a strong predictor of vocal range (Goller & Riede, 2014). Length and area measurements demonstrate that there are pronounced morphological and microstructural differences between the left and right sound generators in a wide range of species. In Bengalese finches, the tissue of the left sound generator is specialized for a shorter vibrating length under high-stress or high-tension conditions, supporting the acoustic findings that this sound generator produces the upper end of their vocal range (Urbano, 2013).

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Coordinating separate physiological systems and the role of HVC

The act of singing requires the precise coordination of separate physiological systems. The respiratory system generates the pressure head driving an air stream that passes through the vocal organ. During quiet respiration, the vocal tissues are relaxed and air passes freely. During phonation, birds assume a position that raises the syrinx in a cranial direction and the dorsal and ventral syringeal muscles are contracted to pull the vocal tissue into the bronchial lumen (adduction). Passing air forces the vocal tissue into a vibratory state that transforms air into sound. Finally, sound is modified in the oro-esophageal cavity with subtle modifications in tracheal length and beak gape width (Podos, Southall, Rossi-Santos, 2003; Joese, Podos, Boetticher & Nowicki, 2000; Riede, Suthers, Fletcher & Blevins, 2006). In the Bengalese finch, it has not been documented that beak gape width is a significant contributing factor in the control of gross acoustic features. However, the respiratory system must generate air pressure in an appropriately timed and carefully regulated manner to initiate and sustain the oscillation of the vocal tissue (Goller & Cooper, 2008). Failing to do so can have an audible impact on the acoustic features of their vocalizations, such as changes in amplitude and/or peak frequency, and once stereotyped sounds can now be perceived as degraded or produced improperly.

Temporal features of song are constrained by the respiratory system. The capacity of the respiratory system limits the duration of vocalization during expiration and the time required to replenish the air supply between sounds. Studies with mockingbirds, known vocal mimics, demonstrated that the capacity for song learning is limited by the physical ability of the species (Zollinger & Suthers, 2004). Juvenile

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mockingbirds were unable to replicate the highly specialized song respiratory pattern of the Waterslager canary, a species capable of singing a trill 4-7 s in duration by taking quick “mini-breaths”. The respiratory motor production of song is not lateralized. During singing, birds activate the left and right expiratory muscles in synchrony. Further, the expiratory and inspiratory muscles are active precisely out of phase with each other. Therefore, there are two separate neural rhythms for controlling expiration and inspiration, but the resultant muscle activity is driven by bilaterally symmetrical neuromuscular activity (Goller & Suthers, 1995a).

Syringeal muscles are unilaterally controlled by the ipsilateral song circuit, however, the respiratory system is bilaterally controlled through respiratory nuclei in the brainstem. In acquiring and rehearsing their song, juvenile songbirds learn to synchronize respiratory and syringeal motor commands in an effective manner. The song nucleus HVC is considered to be a linchpin nucleus in the coordination of these separate motor systems because it is connected to syringeal motor and respiratory motor neurons through RA. Further, contralateral feedback connections to HVC have been established in both RA and brainstem nuclei. Dorsal RA, which innervates RAm also projects back upstream to HVC (Roberts, Klein, Kubke, Wild & Mooney, 2008). RA also projects to the contralateral NXIIIts and RAm, and to the contralateral song circuit through the dorsomedial posterior nucleus of the thalamus (Wild, 1993a). Finally, the network of medullary respiratory nuclei project back to HVC via thalamic nucleus uvaeformis (Wild, 2004).

Electrophysiological recordings have demonstrated that individual HVC neurons will fire only once at precisely the same time-locked instances during song (Hahnloser

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et al., 2002). Multiunit HVC activity typically occurs 45-50 ms prior to the onset of a syllable (Schmidt, 2003). These data suggested that HVC importantly contributes to song timing. To further test this hypothesis, the temperature of HVC was mildly cooled or warmed by fixing a micro Peltier device on the surface of the brain. These temperature manipulations systematically slowed or sped up the song while spectral features remained intact (Long & Fee, 2008). Paired recordings of RA and HVC-RA projection neurons expand the view to suggest that populations of HVC neurons encode individual elements of the song motor pattern, which then fire in a stereotyped sequence, termed a “synfire chain” (Hahnloser, Kozhevnikov & Fee, 2002). Immediate early gene labeling has shown that song encoding likely occurs in populations of HVC neurons organized in a rostro-caudal manner (Stauffer, Elliot, Ross, Basista, Hyson, et al, 2012). Focal HVC lesions only have a transient effect of song as this diffuse organization allows neighbor neurons to compensate ablated cells (Thompson & Johnson, 2007).

What is currently unknown, and of current interest in the songbird field, are the mechanisms HVC utilizes to control the song motor pattern and how much of the motor pattern is dependent upon HVC input. There are currently two models for how song is encoded in HVC. The first states that HVC signals in a feed-forward manner and controls song one subpopulation of RA neurons at a time. That is, for each subpopulation of HVC neurons, there is a downstream population of RA neurons waiting to be activated. Therefore, the song motor pattern can be found in the timing and order of HVC-RA neurons (Long et al., 2010). The second model suggests that HVC encodes critical vocal motor gestures and populations of neurons fire to signal specific physical

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states, or motor gestures of the vocal motor tract (Amador, Perl, Mindlin, & Margoliash, 2013). Electrophysiological recordings of HVC-RA neurons showed the greatest activity just prior to reaching the peak of a movement trajectory; during singing, these peaks typically occurred at the onset of syllables and at instances of large-scale note change. This view suggests that HVC must code sequence properties of song syllables because it is responsible for ensuring the coordination between respiratory, syringeal and oro-esophageal motor systems.

It is proposed that the means to resolving these two models is quantifying HVC-RA activity during active singing and the silent intervals between syllables. The key feature of the motor gesture model, termed Gesture Trajectory Extrema (GTE), is that HVC-RA activity is greater when the bird is phonating, particularly during rapidly changing song features. The feed-forward model makes no such argument and asserts that HVC-RA activity will be in equal proportion throughout the entire song. Picardo et al., (2016) trained adult zebra finches to sing while head-fixed for two-photon calcium imaging. This technique did not allow them to make a distinction between the two classes of HVC projection neurons, however, their analysis of 250 cells in five birds over multiple trials did not show differences in activity across syllables and silent intervals. At present, the most compelling evidence suggests that HVC activity is not coupled to phonation, but rather functions to control song timing.

Chapter 2: Unilateral HVC lesion impairs acoustic features of adult Bengalese finch song

Theories of brain lateralization have existed for centuries, as early as 1700 and 1800s when Franz Gall, Paul Broca, and Carl Wernicke published findings wherein left-hemisphere damage had a critical effect on language production and processing. Around the same time, John Hughlings Jackson presented evidence of right-hemisphere dominance for visuospatial processing. Much of research focused on determining the extent to which cognitive functions could be performed by either the left or right hemisphere alone.

However, neuroimaging research has come a very long way and this technique has demonstrated both sides of the brain are typically active in almost all tasks (Sergent, Ohta & MacDonald, 1992; Price, 2000; Witt, Laird & Meyerand, 2008). This fact, combined with the difficulty of isolating a complex behavior - such as speech or arm reaching - to only one hemisphere, suggests that the truth may be closer to a division of labour that deconstructs a function into its component tasks that are then divided between the left or right hemispheres.

Evidence of hemispheric specialization in humans

Studies on the split-brain patient population have played a vital role in deepening the scientific understanding of lateralized behaviors. The absence of a functioning corpus callosum has enabled scientists to explore the strengths and limitations of each hemisphere on sensory processing and cognitive tasks (Gazzinga, 2000). What has

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emerged from this research is the argument that the corpus callosum is the anatomical feature that allows a multitude of brain areas to evolve into loci of expertise. For example, the increasing complexity of language may have altered the cytoarchitecture of the left inferior frontal gyrus in the process of recruiting neurons for word sequencing and other language functions attributed to Broca's area. As the need for cortical space continued, adjacent neurons could be indoctrinated to support the computational demands. The advantage of devoting local, short fiber networks to computational processes is an increase in processing time and a reduction in metabolic resources. The cost of the recruitment, however, is loss of functions whose cortical areas were co-opted. As a result, both hemispheres potentially become unique experts in complementary tasks and the corpus callosum allows results to be communicated and integrated into a complex, cognitive function.

Hemispheric specialization has been studied in the visual, speech, and motor domains in healthy adult humans. Directed-attention tasks that required focus on either global or local features of visual stimuli suggest that the left hemisphere is active when processing fine details while the right hemisphere is utilized for global features (Fink, et al, 1997). For visual stimuli, Fink et al. (1997) used letters composed of smaller letters as a method of forcing a dissociation between global and local features. When participants engaged in the directed-attention task, PET scans showed strong activation in the right lingual gyrus when focused on global features and left inferior occipital cortex when attending local features. A similar study studied event-related potentials using fMRI and found that in the left hemisphere, the BOLD signal increased in the

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intraparietal sulcus during the presentation of a local cue and in the temporo-parietal region during the presentation of local visual stimuli (Weissman & Woldorff, 2005).

Neuroimaging and testing an array of language-related functions has uncovered many more regions outside of Brodmann's area 44 and 22 and the primary motor cortex as areas important for articulation. In the process, it has also revealed that the right hemisphere plays a critical role in processing prosody, or the underlying emotional content of speech (Price, 2000; Gandour et al, 2004; Witteman et al, 2012). Right hemisphere lateralization for prosody and left hemisphere activation for word meaning has also been demonstrated in children, indicating specialization is a process that goes hand-in-hand with language learning. Auditory areas in the right hemisphere were found to be engaged across multiple studies, suggesting that emotional content may be processed primarily from acoustic features (Witteman et al, 2012). Right hemisphere specialization for prosody can be moderated by the subject's primary language, as Chinese speakers also utilized left hemisphere areas when processing word meaning in addition to the emotional content of the acoustic signal (Gandour, et al., 2004).

Handedness is a particularly well-known example of lateralized motor control. It was established early on that nearly all individuals preferred the use of one arm – the “dominant” arm – over the other and that there were marked differences in speed, precision and fine motor control. Reasons for the differences have been explored over decades of research. Early theory accepted that the hemisphere contralateral to the “dominant” arm held a robust network for motor control and that the nondominant hemisphere-arm system simply held a weaker analogue. Eric Roy and colleagues noted, however, while the dominant hemisphere-arm system in right-handed subjects,

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excelled at adjusting trajectory based on visual and proprioceptive feedback, the nondominant hemisphere-arm system had faster reaction times (Roy, 1983; Roy, et al., 1994). They proposed that the left hemisphere processed feedback control and the right hemisphere specialized in feedforward control in right-handed individuals. Recent research elaborated upon this idea and demonstrated that participants relied on predictive strategies for moving the right arm and a learned “spatial” position for directing the left arm (Mutha et al., 2013).

Lateralization of motor production in Bengalese finch

As introduced in Chapter 1, the vocal tissues in Bengalese finch males and females are highly asymmetric. As microstructural asymmetries are not consistent between sexes, only male vocal tissue will be discussed further. In the male syrinx, there is evidence that the left sound source is highly specialized for producing their upper frequency range. Endoscopic recordings of vocal tissues in phonating crows indicate that the lateral labium typically occludes most of the bronchial aperture during phonation (Goller & Larsen, 1997). Despite this, we found that the left lateral labium of the Bengalese is nearly a one-third the size of the right lateral labium (Urbano, 2013). Microstructurally, compared to the right medial and lateral labia, the left vocal tissue has a greater concentration of extracellular matrix (ECM) components – hyaluronic acid, collagen and elastin – that are critical for maintaining higher rates of vibration as well as protecting the tissue from stress and shear forces (Chan et al., 2007). Shorter vibratory lengths are necessary for producing the upper frequency range and its smaller size undergoes greater deformation to cover the bronchial aperture.

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Similar to the left sound source, the right vocal tissue are specialized for broadband sound. It has a large deep layer composed entirely of hyaluronic acid. The absence of structural ECM components in a thick tissue mass reduces fine neuromuscular control as it is improbable that oscillations migrating outward will continue to remain in phase. Noisy, highly entropic sound is generally produced by multiple layers vibrating at different rates and amplitudes.

Given the drastic anatomical differences in the vocal organ, it is entirely possible that unique neural networks evolved in the left and right song systems. Strongly arguing in favor of this possibility, we have also determined that the Bengalese finch wild-type ancestor, the white-rumped munia (*Lonchura striata*), is anatomically similar, yet limited in its vocal range. Unlike the Bengalese finch, muniyas rarely produce higher frequency syllables at a population level (Kagawa, et al., 2012). Although our findings were limited to morphological asymmetries, we can confidently say that the left lateral labium is significantly smaller than the right lateral labium (Urbano, 2013). Generations of domestication and breeding for parenting skills may have had unintended consequences on song quality and resulted in highly lateralized vocal abilities unique to the modern Bengalese finch.

Bengalese finches are considered to be left-hemisphere dominant, similar to Waterslager canaries. Ablating HVC in the left hemisphere (Okanoya, Ikebuchi, Uno, & Watanabe, 2001) impaired their ability to discriminate between conspecific songs. Additionally, left NXII's resection destroyed the acoustic quality of the bird's contact calls (Okanoya & Yoneda, 1995). However, given strong anatomical asymmetry at the level of the vocal tissue, it begs the question whether these previous studies provide

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sufficient information to support the claim that Bengalese finches are left-hemisphere dominant. Dominance implies control is allocated to one hemisphere whereas lateralization argues that task control is divided between hemispheres. For example, an alternative explanation is that the left hemisphere is critical for the production of higher-frequency syllables and the right hemisphere is equally critical for lower frequency syllables. Experiment 1 proposes to shed light by addressing changes in acoustic and temporal features after unilateral left or right HVC lesions of varying magnitude. We predict that damage to left HVC will lower song peak frequency and damage to right HVC will affect song entropy.

Methods

Subjects

28 male Bengalese finches were purchased from local suppliers and housed in communal flight cages until the start of the experiment. Birds were kept on a 14:10 light:dark cycle during housing and experimental testing. They were fed seed *ad libitum*, supplemented with vitamins and vegetables.

Housing procedures

During data collection, birds were kept in individual cages (31.8×10.5×25.4 cm) and the microphone was centered 14 cm above the perch.

Pre- and Post-Surgical Procedures

Birds were deeply anesthetized by vaporizing isoflurane (1-2%) in breathing air (flow rate, 2 L/mg). Surgical anesthesia was confirmed by an absence of physical

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movement following a light pinch on one of the bird's talons. Birds recovering from surgery were kept in individual cages and monitored until they perched.

Electrolytic HVC lesion

To enable precise variation in lesion extent within HVC, the electrolytic lesion technique was used. After birds were deeply anesthetized, feathers were removed to expose the ears and scalp. Birds were placed on a stereotaxic frame and secured with ear bars. An incision was made along the anterior to posterior axis, parting the skin and exposing the skull. Using the mid-sagittal sinus as the point of origin, the distinctive electrophysiological activity of HVC neurons was used to identify the mediolateral and dorsoventral boundaries of the nucleus. Multiunit neural activity was recorded using a differential AC amplifier (10 kHz low-pass, 100 Hz high-pass, 10,000 gain; Model 1700, A-M Systems, Sequim, WA) and a tungsten, parylene-c insulated electrode (shaft diameter: 0.081 mm, tip diameter: 2-3 μm , Microprobes, Gaithersburg, MD). Table 1 outlines the electrolytic lesion parameters for micro- and full lesions. Then, the exposed area was covered with vacuum grease and the skin was sutured closed. An equal number of sham surgeries performed wherein birds undergo the surgical procedure with the electrode inserted for equal duration without passing current.

Table 1. Lesion parameters for sham, micro- and full lesion conditions.

Condition	# sites	Current duration	Current amplitude
Sham	6	0	0
Micro lesion	3	30 s	100 μA
Full lesion	6	60 s	100 μA

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Acoustic recording procedure and data acquisition

Birds were recorded for 1-2 days before surgery. After surgery, they were recorded continuously for seven days. Automatic recording software continuously buffered three seconds of data until triggered by sound amplitude exceeding a user-defined level. Microphone recordings were amplified and high-pass filtered (300 Hz; RME audio, Haimhausen, Germany) and digitized (44.1 kHz sample rate, 16 bit resolution) via an analog-to-digital converter (National Instruments, NI USB-6251, Austin, TX), and saved to disk on a computer running Avisoft Recorder software (Avisoft Bioacoustics, Berlin, Germany).

Acoustic Analyses

Song. Using Avisoft SASLab (Berlin, GE) automatic measurement function, we measured duration, intersyllable interval (ISI), mean peak frequency, and amplitude of individual song syllables. Duration (s) is the onset to offset of each song syllable, and the ISI is the offset of the preceding syllable to the time of the onset of the subsequent syllables (s). Mean peak frequency is the frequency produced at the highest amplitude (kHz). Mean amplitude is the measurement (decibels, dB) of the average of the syllable. Entropy is a measure of the randomness of sound with a range from 0 (sine wave) to broadband (1).

Contact calls. Using the same methods as Urbano (2013), contact calls were visually identified and analyzed. Using a fast-Fourier transform in SASLab, amplitude, peak frequency, bandwidth, and the percent of acoustic energy below 2.2 kHz was measured. Bandwidth is the difference between the minimum and maximum acoustic frequency of the call. Larger bandwidth indicates a call with frequency modulation or

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noisy, broadband sound. The percentage of acoustic energy measures the relative proportion of sound that falls below 2.2 kHz. Greater proportions indicate a predominately lower frequency, right-side generated vocalization (Urbano, 2013).

Statistical analyses

Song was not always available for all animals for all seven days. Occasionally, a male did not sing until 48-72 hours after lesion. For two subjects, data from post-surgery days five and six were contaminated by 60 Hz background noise and could not be used. Statistical analyses were performed only on days with the most number of animals.

Regression.

For left and right HVC lesion groups, individual regression analyses were calculated using the lesion extent (micrometers of HVC tissue damaged) as a predictor variable for the change in mean peak frequency, amplitude and entropy. The change in mean peak frequency, amplitude and entropy were calculated as a percentage change compared to presurgery song.

One-way ANOVA. Data from post-surgery day 7 was used to perform between-groups comparisons for syllable peak frequency, entropy, amplitude, duration, and intersyllable interval. All results are reported as mean \pm SEM.

Within-subjects t-test. Contact calls from pre-surgery and post-surgery recordings were compared for differences in amplitude, peak frequency, bandwidth and the percent of acoustic energy below 2.2 kHz.

Histology

After data were collected, animals were euthanized via overdose of anesthetic isoflurane. The brain was extracted and stored in formalin until further processing.

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Brains were embedded in Tissue-tek (Sakura Finetek USA Inc., Torrance, CA) and sectioned at 50 μm thickness. Sections were photographed and the boundaries of lesion damage and contralateral HVC were outlined in Photoshop (Adobe Systems, San Jose, CA). For each section containing HVC, ipsilateral and contralateral hemispheres were overlaid to measure the percent of HVC damaged.

Results

Lesion verification

6-99% of HVC was ablated, with a mean of $52.05\% \pm 6.00$ (Figure 7). There were no differences between hemispheres in either the micro ($p > 0.1$) or the full lesion ($p > 0.1$) groups.

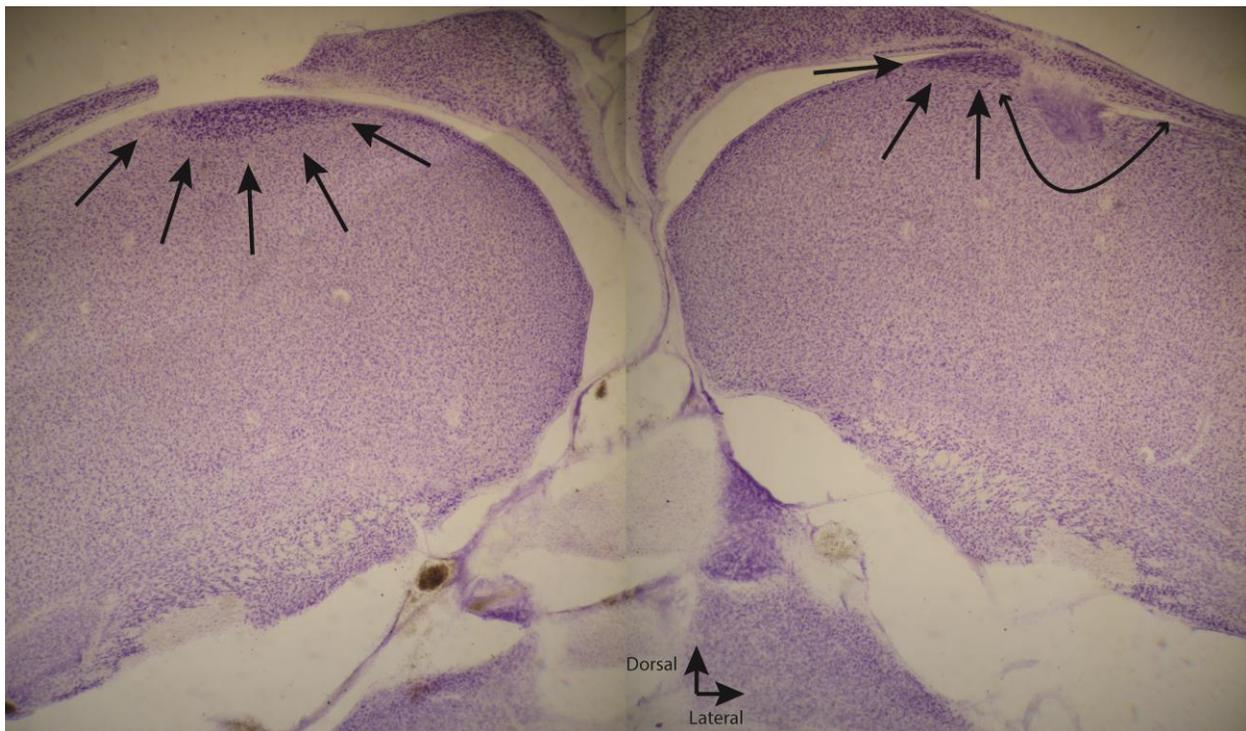


Figure 7. Coronal cross section of a unilateral HVC lesion. 50 μm section stained with cresyl violet. Intact (*left side*) HVC and partial (*right side*) HVC boundaries are marked with arrows. Ventral extent of lesion damage is outlined with a black bi-directional arrow.

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Lateralized HVC damage predicts changes to song peak frequency amplitude and entropy

Left HVC lesion extent was a marginally significant predictor of change in peak frequency ($F(1,6) = 5.64$, $p < .056$), but not for the change in mean peak amplitude or entropy ($F(1,6) = 2.49$, n.s.; $F(1,6) = 0.17$, n.s., respectively). Right HVC lesion extent was not a significant predictor of mean peak frequency ($F(1,7) = 2.40$, n.s.), but it was a significant predictor of change in mean peak amplitude ($F(1,7) = 10.88$, $p < .02$) and mean entropy ($F(1,7) = 14.91$, $p < .007$).

HVC-lesioned groups produce fewer higher frequency syllables

In a subset of animals, we measured the proportion of higher frequency and lower frequency syllables. The left and right full lesion groups show an increase in lower frequency syllables and a reduction in higher frequency syllables at PSD7 (Table 2).

Table 2. Percent increase and decrease in syllable type at PSD7

		PSD7
Right HVC lesion	Lower frequency	37.26% ± 16.10
	Higher frequency	-56.48% ± 17.80
Left HVC lesion	Lower frequency	27.85% ± 8.89
	Higher frequency	-77.77% ± 10.72

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HVC lesions do not alter spectral or temporal features of learned contact/distance calls.

Within-subjects t-tests showed no differences between pre-surgery and post-surgery calls for amplitude, peak frequency, bandwidth and the percent of acoustic energy below 2.2 kHz (Figure 8).

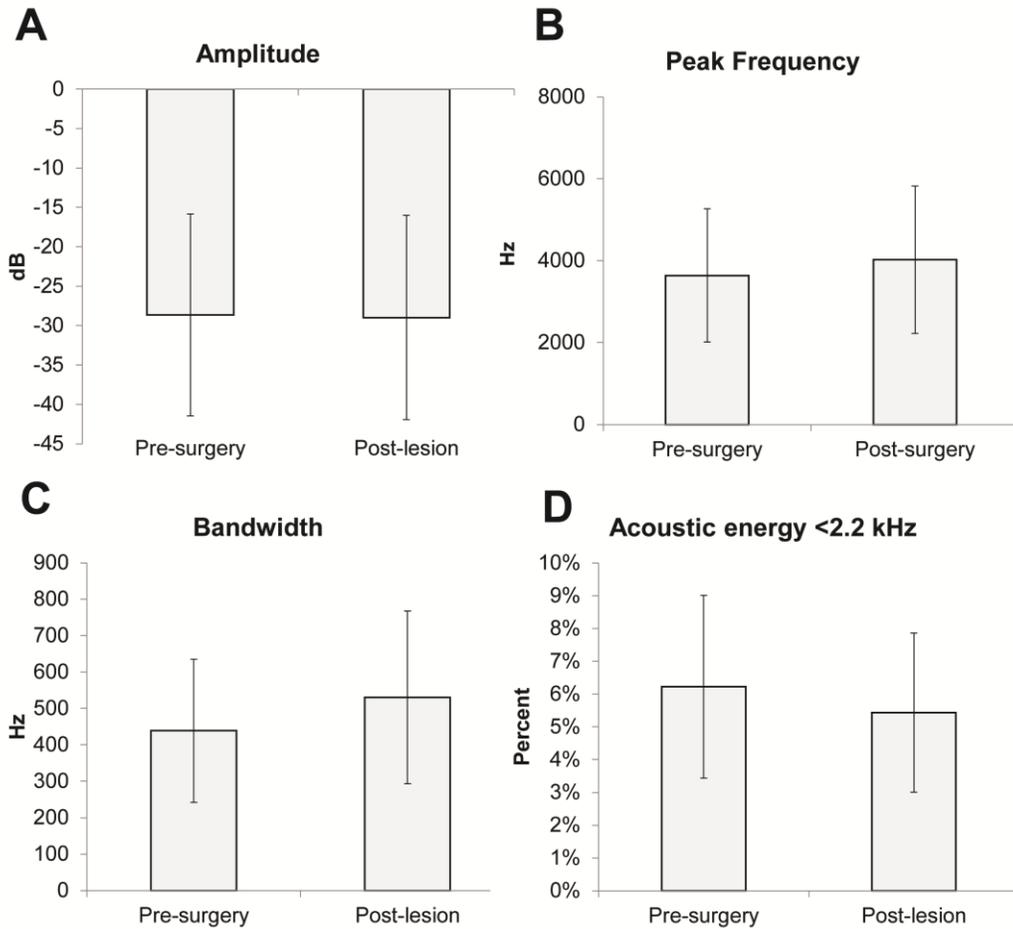


Figure 8. Contact calls are not altered by HVC damage. Bar graphs comparing pre-surgery and post-surgery contact calls for a) amplitude, b) peak frequency, c) bandwidth and d) acoustic energy < 2.2 kHz. Mean \pm S.E.

Between groups comparison at post-surgery day 7

Large left HVC lesions exhibit greatest decline in peak frequency. A one-way ANOVA for peak frequency at PSD7 resulted in significant between-groups differences ($F(4,27) = 15.714$, $p < 0.001$, Figure 9). The mean peak frequency of all lesion groups was significantly lower than sham animals ($M \pm SEM$: $4.20\% \pm 4.00$). Left (-12.18% \pm 4.86) and right (-14.78% \pm 3.11) micro-lesion groups were similar and significantly lower than full lesion groups. Left (-45.04% \pm 8.19) was marginally lower than right (-31.66% \pm 2.25, $p = 0.06$), providing evidence that left HVC plays a critical role in regulating higher frequencies.

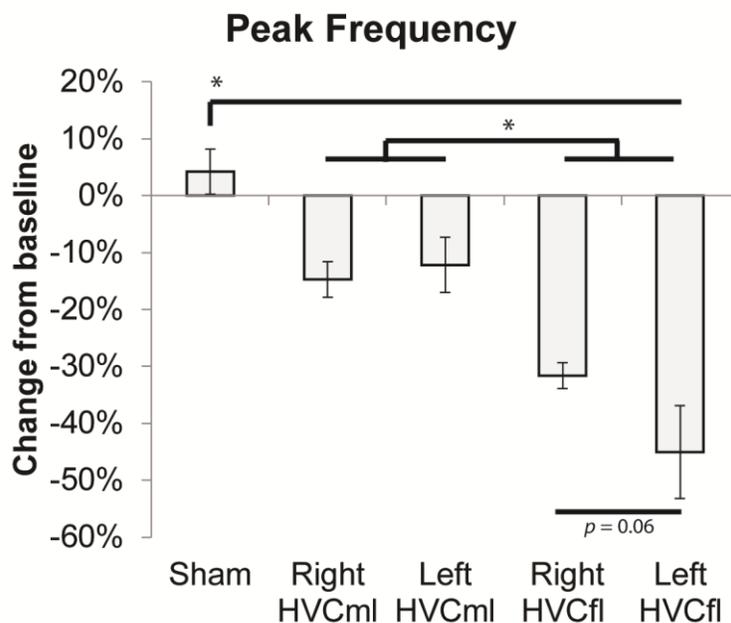


Figure 9. HVC lesions reduce peak frequency. Peak frequency is lower in all experimental animals. Full lesions show the greatest reduction, with left lesions significantly lower than all other experimental groups. Mean \pm SEM. Asterisk denotes $p < 0.05$ to $p < 0.005$.

Large HVC full lesions increase song entropy. A one-way ANOVA for entropy at PSD7 resulted in significant between-groups differences ($F(4,27) = 4.860$, $p < 0.006$, Figure 10A). Mean entropy of all lesion groups except the right micro-lesion group

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(3.64% ± 5.95) was significantly higher than sham animals (-3.01% ± 1.97). Left (17.44% ± 11.09) and right micro-lesion groups were similar; however, the entropy of full lesion groups was significantly higher than the right micro-lesion group.

Individual variability influences amplitude decline. A one-way ANOVA for amplitude at PSD7 resulted in significant between-groups differences ($F(4,27) = 7.204$, $p < 0.001$, Figure 10B). Mean amplitude of all lesion groups except the right micro-lesion group (-7.99% ± 11.42) was significantly lower than sham animals (5.48% ± 3.74). There were no differences between left (-2.83% ± 1.20) and right micro-lesion groups. There were also no differences between left (-46.89% ± 6.92) and right (-29.25% ± 7.75) full lesion groups.

Differences between micro- and full lesion groups were not influenced by lesion size. Right micro-lesions were significantly lower amplitude than left ($p < 0.004$) full lesions, but not right full lesions ($p = 0.09$). Left micro-lesions were not significantly lower amplitude than either left ($p = 0.12$) or right ($p = 0.94$) full lesions.

HVC lesions do not produce a systematic change in syllable duration. A one-way ANOVA for syllable duration recorded at PSD7 did not reveal significant differences between lesion groups ($F(4,27) = 0.410$, $p = 0.800$, Figure 10C).

HVC lesions increase silent gaps between syllables. A one-way ANOVA for inter-syllable interval at PSD7 resulted in significant between-groups differences ($F(4,27) = 5.495$, $p < 0.003$, Figure 10D). The ISI of all lesion groups except the right micro-lesion group (6.66% ± 12.09) were significantly longer than sham animals (-14.40% ± 9.36). Left (43.82% ± 12.27) and right micro-lesion groups were not significantly different from each other. Left (61.71% ± 21.77) and right (51.46% ± 12.61)

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full lesion groups were also similar. However, while the ISI of the right micro-lesion group was shorter than both left ($p = 0.02$) and right ($p = 0.054$) full lesion conditions, left micro-lesion animals were not significantly different from either full lesion group.

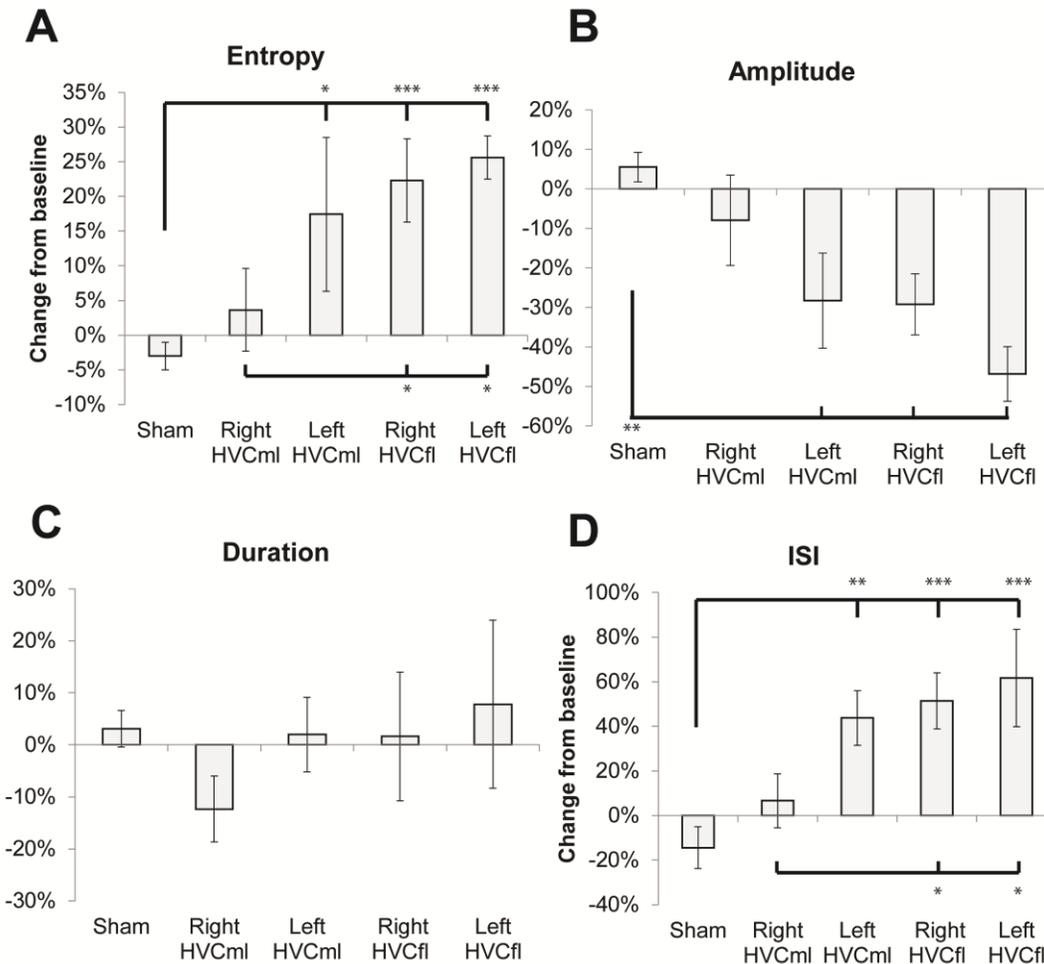


Figure 10. Between-groups comparison at post-surgery day 7. Bar graphs of group means for a) entropy, b) amplitude, c) duration and d) ISI. Typically, right HVC microlesions were not different from sham lesions and left HVC microlesions were not different from full lesion conditions. * $p < 0.05$, ** $p < 0.01$, * $p < 0.005$**

Summary and Discussion

All experimental animals showed evidence of vocal impairment. HVC damage also suppressed song production. Three sham animals and two experimental animals

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did not sing on the first day following surgery. Another two experimental subjects did not sing until the third day. Initial songs following HVC lesion were extremely soft and similar to juvenile subsong; it is also possible that songs from the first few days were not detectable with the microphone recording. It has been reported that bilateral HVC lesions in canaries can result in “silent song”. HVC lesions produced song deficits that varied from subject to subject. Syllables were much more stable at post-surgery day 7 and it was apparent that HVC damage had a lasting effect on several acoustic and temporal features.

Left HVC specialization for higher frequency syllables

We did not see evidence that cleanly dissociates the effects of left and right lesions. However, animals in the left full lesion group stood out in three measures. Higher frequency syllables were nearly eliminated and replaced by novel lower-frequency syllable types in the left HVC lesion group (Table 2). Syllable peak frequency in the left full lesion group was the lowest of all conditions, marginally lower than even the right full-lesion group (Figure 9). Last, while mean entropy was not significantly different between left and right lesion conditions, the absence of higher frequency components had a greater impact on the entropy of left-lesioned song. Together, these results support the possibility that producing higher frequency syllables in a species-typical manner relies heavily on intact left HVC circuitry.

Slight song deficits in sham-lesioned animals

It has been reported that electrode insertion can result in song deficits (Okanoya, 2004). In all measures, sham animals showed slight changes due to the mechanical damage of lowering and retracting the electrode. However, there were no differences

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between left- and right- HVC shams and histology did not find evidence of more than 50 μm of tissue damage. Combined with our within-subjects experimental design, I propose changes in the sham group does not negatively affect our findings. We performed additional sham surgeries and confirmed the decline in peak frequency is HVC-specific (see Appendix C).

Potential evidence for left-hemisphere dominance in song production

Interestingly, there is a consistent trend that involves the left microlesion group. In entropy, amplitude and ISI, the left microlesion is nonsignificant when compared to the full lesion conditions. Qualitatively, spectral and temporal changes after left microlesion are greater than the effects of a right microlesion, even though they did not reach statistical significance. Paired with evidence that left HVC is specialized for higher frequency syllables, I propose that either higher frequency syllables, which are often produced as trills, are a defining characteristic of Bengalese finch song – and their absence is noticeable – or that left HVC plays a slightly stronger role in song production in this species. The first instance would imply that ablation of HVC neurons that encode higher frequency syllables inherently alter song sequence with the removal of these syllables. The second possibility is that Bengalese finches rely more on left HVC to maintain species-typical song rhythm and syllable stereotypy. These two possibilities are not mutually exclusive.

Individual variability and effects on duration and intersyllable interval

The temporal effects of HVC lesions were not systematic on a syllable-to-syllable level. Even at post-surgery day 7, although song was much more stable than the initial two-three days, duration effects were highly variable. Some syllables were shorter than

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baseline and others were identical. Some individuals produced abnormally long syllables or potentially combined two syllables into one vocalization. Left lesioned animals often inserted noisy, broadband syllables of varying duration. Both right and left-lesioned animals inserted brief, abnormal silent intervals between “incomplete” syllables. It is difficult to conclude whether these intervals are genuine inspirations, unvoiced segments, or were simply too soft for recording. Of note were abnormally long, broadband sounds that were inserted by both left and right lesion groups. Two left lesion individuals produced syllables with overlapping entropic and harmonic features of varying duration and fundamental frequency, suggesting their song production greatly suffered from poor motor coordination.

In contrast, it is evident that there are abnormally long silent intervals between syllables, especially in the full lesion and left HVC microlesion groups. Longer silent intervals could be an indicator that HVC damage resulted in a systematic delay in motor commands to RA, midbrain nuclei, and thalamus. Another possibility is that HVC damage has impaired motor control of phonation. The latter is more likely as it would also explain the variability in syllable duration and poor syllable production.

Chapter 3: Acoustic, but not respiratory, features recover after unilateral HVC lesion

Two trends emerged from the findings in chapter 1: a) left HVC may be more critical to song maintenance than right HVC and 2) HVC lesions produce temporal changes that are not lateralized. In this chapter, we explore the possibility of hemispheric dominance for song learning and memory by directly comparing left and right lesion conditions and tracking song recovery over multiple timescales. In a separate study, we study the underlying song respiratory motor pattern and similarly track recovery of respiratory features over time.

Hemispheric dominance for speech develops over time

Devoting localized circuits to increase processing speed is an advantage that provides a compelling argument for large-scale brain networks underlying complex behaviors. However, it does not offer an opinion on where related memory or a basic template is stored. For this and other reasons, the possibility of hemispheric dominance for speech and language remain relevant today.

A left hemisphere bias for language appears in children as young as two months. Behaene-Lambertz and colleagues have demonstrated that two-month-old infants orient towards a playback of their native language over others (Behaene-Lambertz & Houston, 1998). An fMRI study showed activation of the left inferior frontal lobe, which contains Broca's area, in two and three-month olds listening to speech stimuli (Dehaene-Lambertz, Dehaene, & Hertz-Pannier, 2002).

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Lateralization in children varies with age. Holland et al., (2001) conducted an fMRI study with typically developing children between the ages of 7-18 during a word-fluency task. Their results showed left lateralization in all subjects, but the strength of lateralization is correlated with age ($r = 0.63$). Functional transcranial Doppler ultrasound measuring blood flow from the middle cerebral arteries in children aged 1-5 showed complementary results. Older children measured during a naming task were strongly left lateralized whereas younger children were more likely to exhibit variable response (Kohler, et al., 2015).

These studies suggest that areas tied to speech and language in adults are already engaged prior to vocal exploration. Further, increasing left lateralization as children age suggests that language development increases the use of left-hemisphere brain areas. If lateralization of function is a key component for the evolution of speech, then something similar could also exist in vocal learning animals, for example the songbird.

Evidence of lateralized learning and memory in songbirds

Manipulating auditory feedback in juveniles and adults indicate that song production is actively guided by an internal template or error-correction process (e.g. Woolley & Rubel, 2002; Tumer & Brainard, 2007; Sober & Brainard, 2009; Andalman & Fee, 2009; Williams & Mehta, 1999; Urbano, et al., 2013). This begs the question: where is this template stored?

Caudomedial nidopallium (NCM). The oscine secondary auditory regions are forebrain nuclei that receive input from primary auditory areas nucleus ovoidalis and Field L (analogous to mammalian primary auditory cortex). These secondary areas are

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upstream to the song motor and song learning systems and indirectly influence the song system. NCM is the most likely candidate for containing the tutor song. Immediate early gene expression (IEG) in late-stage juvenile zebra finches (approximately 56 dph) is particularly robust after exposure to tutor song, as opposed to novel conspecific song or silence (Gobes, et al., 2010). NCM lesions also eliminate natural preference for tutor song in adult males without disturbing song production (Gobes & Bolhuis, 2007). IEG downregulation in juveniles selectively impaired tutor song imitation (London & Clayton, 2008).

In zebra finches, tutor song playbacks elicited greater IEG expression in left hemisphere NCM in awake juveniles and adults (Moorman, et al., 2012). IEG immunocytochemistry and fMRI studies have also demonstrated the strength of song learning is associated with greater left hemisphere NCM activity (Moorman, et al., 2015; Van Der Kant, et al., 2013). Lateralized engagement of NCM would suggest that the tutor song is encoded in the left hemisphere.

HVC. Electrophysiological and IEG responsiveness of HVC neurons to tutor song exhibits developmental changes. In unanaesthetized 35-69 dph juvenile zebra finches, HVC neurons show preferential firing - relatively higher rates of activity - in response to tutor song as opposed to their own song (bird's own song, BOS), conspecific song, heterospecific song or white noise (Nick & Konishi, 2005a). Disrupting HVC activity in 40-53 dph zebra finches while the tutor was singing via optogenetic or electrical stimulation was sufficient to prevent song and syllable imitation, respectively (Roberts, et al., 2012). In adult songbirds of several studied species, HVC neurons prefer BOS to tutor song or conspecific song (Margoliash & Konishi, 1985; Margoliash, 1986; del

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Negro et al., 2005). IEG expression in response to tutor song also largely absent in adult males (Boluis et al., 2000, 2012). It's highly possible that HVC may contain the template for the bird's own song and plays a critical role in online error correction.

HVC's involvement in singing and song perception have been investigated in depth. There is little evidence of lateralization in singing and most evidence points toward "switching" between the two hemispheres in zebra finches (Wang, et al., 2008; Long & Fee, 2008; Schmidt, 2003). Unilateral lesions with Waterslager and domestic canary (*Serinus canaria forma domestica*) strains have shown left HVC damage produced greater song impairment (Nottebohm, 1976; Halle, et al., 2003).

Evidence of lateralized song perception is similarly mixed. Only three studies have examined lateralized HVC activity using BOS. Poirier et al (2009) reported greater right HVC activity in response to BOS playback. Left HVC neurons in awake starlings are highly responsive to song features signaling individual identity, but there was no evidence to suggest especial response to BOS and anesthesia eliminates lateralized activity (George, et al., 2005b; George, et al., 2005a). In awake zebra finches, there was greater IEG expression in left HVC in response to playback, but IEG expression did not discriminate between tutor song, novel song, or silence (Moorman, et al., 2012). A follow-up study in sleeping zebra finches produced different results: right HVC was more active than left (Moorman, et al., 2015). At present, while there is strong evidence to suggest HVC encodes BOS, direct comparison of left and right HVC activity during BOS playback requires further study.

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Ontogeny of the song respiratory motor pattern

Subsong is characterized by softer, unstereotyped vocalizations that are largely similar to one another. These initial vocalizations, termed “protosyllables”, develop complex acoustic and temporal features that serve as unique syllable identifiers in the adult song. Similarly, the underlying air sac pressure pattern of subsong is lower amplitude and can share gross similarity across song-related expiratory pulses (EPs; Veit, et al., 2011; Cooper et al., unpublished observations). Unlike adult EPs – which have a 1-to-1 relationship with song syllables – subsong EPs can contain multiple syllables, or be partially filled with silence. Some syllables, often in the lower range of EP amplitude, do not contain syllables at all (Veit et al., 2011; Cooper et al., unpublished observations). At adulthood, all subjects exhibited the normal EP-syllable relationship, suggesting that constant rehearsal during the sensorimotor and plastic phases serve to improve the coordination between respiratory and syringeal motor systems.

Rapid song recovery in the Bengalese finch

Bengalese finch males learn one song during the sensorimotor phase and as adults, sing that song for the rest of their life (Cooper et al., 2012). Despite the seeming stereotypy and simplicity of their song repertoire when compared to other studied species (e.g., the northern mockingbird, gray catbird, and brown thrasher), the Bengalese finch relies heavily on auditory feedback for song maintenance. Deafening studies have demonstrated that compared to the chaffinch and the zebra finch, song deteriorates within days (Woolley & Rubel, 1997; Yamaguchi & Okanoya, 1997). Reversible deafening using ototoxic lesions that selectively destroy inner ear hair cells

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show a time-matched recovery of both hearing and song quality (Woolley & Rubel, 2002). Further, using miniature headphones to replay the bird's song with a shift in the fundamental frequency causes the male to compensate for the perceived difference in the frequency shift compared to their typical song (Sober & Brainard, 2007).

Given the role of HVC in producing adult song and its candidacy for the BOS template, and the species' propensity for online error correction, we explored lateralization of song recovery by comparing the effects of left and right HVC lesion. There is already some evidence that Bengalese finches may be left-hemisphere dominant. Bengalese finches are considered to be left-hemisphere dominant, similar to Waterslager canaries. Ablating HVC in the left hemisphere (Okanoya, Ikebuchi, Uno, & Watanabe, 2001) impaired conspecific song discrimination in Bengalese finches compared to similar damage to the right HVC. Okanoya has also reported that left HVC lesions have a greater impact on song quality compared to lesions of equal magnitude in right HVC, a finding that we have replicated and extended in chapter 2.

Methods

Animals

Data acquired from seven days of recording twenty-two animals in Experiments 1 and 2 (Chapter 2) are used in this study for comparing the rate of song recovery (Table 3). Post-surgery month 1 recordings were collected in a subset of nineteen animals (left = 6; right = 6, sham = 5). Post-surgery month 5 (PSM5) recordings were collected from eight full lesion animals (left = 4; right = 4). Acoustic and air sac pressure data were

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recorded in eight animals were before and after lateralized HVC damage (Experiment 3: left = 4; right = 4).

Table 3. Group size for measure of song recovery.

	Left HVC	Right HVC
Microlesion	4	4
Full lesion	5	5
Sham	4	

Housing procedures

During data collection, birds were kept in individual cages (31.8×10.5×25.4 cm) and the microphone was placed 14 centimeters centered above the perch.

Surgical procedure.

Pre and post-operative general surgical procedures, HVC mapping and lesion parameters are described in Chapter 2 methods.

Air Sac Cannulation. Anesthetized birds will be placed in a supine position. A small opening was made just below the rib cage allowing for insertion of silastic tubing (6.5 cm length, 0.76 mm I.D.; Dow Corning, Midland, MI) into a posterior thoracic air sac. The cannula was then sutured to the third rib to minimize cannula movement. The free end of the tube was attached to a custom-built piezo-resistive pressure transducer (Fujikura, FPM 02PG; Santa Clara, CA) that was attached to a Velcro tab centered on the bird's back and held in place by a small elastic band wrapped around the bird's thorax.

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Recording procedure

Song and air pressure were recorded continuously and data were saved to disk when sound amplitude or respiratory pressure amplitude exceeded a user-defined level longer than 10 ms.

Single-channel microphone recordings. Microphone recordings were amplified, high-pass filtered (300 Hz; RME audio, Haimhausen, Germany) and digitized (44.1 kHz sample rate, 16 bit resolution) via an analog-to-digital converter (National Instruments, NI USB-6251, Austin, TX).

Multi-channel recordings. Microphone and air sac pressure were recorded simultaneously. Data were saved to disk in .wav file format on a computer running Avisoft Recorder software (Avisoft Bioacoustics, Berlin, Germany). Air sac pressure signal was amplified 100x and low-pass filtered (6 kHz cutoff, Brownlee Precision, Model 440, Santa Clara, CA). The recordings were digitized (22.05 kHz sample rate, 16 bit resolution).

Data analysis

Two to seventy-five songs (depending on singing frequency of the individual bird) were manually selected from each timepoint. To quantify rate of song recovery, two segments of songs produced each day for seven days after surgery were selected. Data for two animals were contaminated by 60 Hz noise during post-surgery days 5 and 6 and could not be used. Full lesion animals continued to be recorded once a month for 6 months to evaluate slow-changing spectral and temporal variables. One animal died before PSM 6; therefore, we performed group comparisons of acoustic parameters at PSM 5. As repeated air sac cannulation leads to rapid deterioration of pressure

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recordings, it was only performed at PSM 6. In the case of one animal, we did not get stable air sac pressure recording and we repeated the cannulation at PSM 8.

Acoustic Analysis of Song. Using the methods described in Chapter 2 methods, we measured song syllable duration, intersyllable interval (ISI), mean peak frequency, and amplitude.

Song Similarity Analysis. The song similarity before and after lesion will be measured using the procedures described in Urbano et al. (2013). Briefly, 5-10 two-s song segments were manually selected from the beginning of song bouts within each timepoint. Using Sound Analysis Pro (Tchernikovskiy et al, 2000), pre-surgery song segments were compared to segments from post-surgery day three (PSD3) and seven (PSD7). The song similarity function uses five acoustic parameters (pitch, frequency modulation, amplitude modulation, Weiner entropy, and goodness of pitch) and compares two sound files along a 10 ms sliding window to identify overall acoustic similarity between songs. Scores range from zero to 100 percent similarity.

Air Sac Pressure Analysis. Using custom-written LabVIEW software, we segmented expiratory and inspiratory pulses produced during song. Expiratory pressure was defined as supraatmospheric pressurization and inspiration subatmospheric pressurization. The mean amplitude and duration for expiratory and inspiratory pulses were calculated. The expiratory and inspiratory pulse slope was calculated during the first 7 ms of the supratmospheric pressurization of the expiratory pulse of air. Air pressure amplitude of expiratory and inspiratory pulses were calibrated using a vertical water column. The voltage output of the pressure transducer was measured from 1 cm to 20 cm H₂O, using 2 cm H₂O step intervals. A linear function

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was determined for the transducer used for each bird, and this was used to transform all air sac pressure voltage values.

Statistical analysis

Mixed-model repeated measures ANOVA for song similarity. Song was not always available for all animals for all seven days. Statistical analyses were performed only on days with data from all animals. Micro- and full lesions were analyzed separately to compare rates of recovery between left and right hemisphere lesions.

One-way ANOVA. Data from post-surgery month 1 was used to perform between-groups comparisons for song similarity, syllable peak frequency, entropy, amplitude, duration, and intersyllable interval.

Between-subjects t-test. Data from post-surgery month 5 was used to compare left and right HVC lesion groups for song similarity, syllable peak frequency, entropy, amplitude, duration, and intersyllable interval.

Within-subjects t-test. Expiratory pressure duration, amplitude and slope for contact calls were compared separately across pre-surgery and post-surgery recordings.

Repeated-measures ANOVA for respiratory features. Expiratory and inspiratory pressure duration, amplitude and slope were compared separately across pre-surgery, post-surgery and 6-8mos post-surgery song. All results are reported as mean \pm SEM.

Results

Similarity scores for microlesions and shams are not significantly different

A 3x3 (condition x timepoint) mixed-model repeated measures ANOVA for song similarity was significantly different across timepoints ($F(2,18) = 12.327$, $p = 0.000$, Figure 11A). HVC damage reduced similarity score in all animals. We did not find a significant interaction effect ($F(4,18) = 0.505$, $p = 0.733$).

Lateralized rates of song recovery are observed in full lesion conditions

A 3x3 (condition x timepoint) mixed-model repeated measures ANOVA for song similarity was significantly different across timepoints ($F(2,24) = 56.221$, $p = 0.000$, Figure 11B). There was also a significant interaction effect ($F(4,24) = 3.238$, $p = 0.029$).

Similarity decreased after HVC lesion. In all groups, there was a significant decline in mean similarity score after surgery (PSD3: $p = 0.000$; PSD7: $p = 0.019$).

HVC damage reduces song similarity. There were group differences in the baseline data. Pre-surgery songs in the sham condition were significantly “more similar” than pre-surgery songs in either lesion condition (Figure 11B). However, sham groups (58.27 ± 6.80) had higher similarity scores at post-surgery day 3 than either lesion group (Right HVC lesion: 22.58 ± 5.55 ; Left HVC lesion: 10.77 ± 6.08).

Songs are slower to recover after a left HVC lesion. At post-surgery day 7, shams (55.40 ± 8.63) and right HVC lesion (40.952 ± 7.05) were not significantly different, showing moderate recovery in the right lesion condition. However, the left lesion condition (18.68 ± 7.72) was significantly lower than either the sham ($p = 0.008$) or right lesion ($p = 0.054$) conditions.

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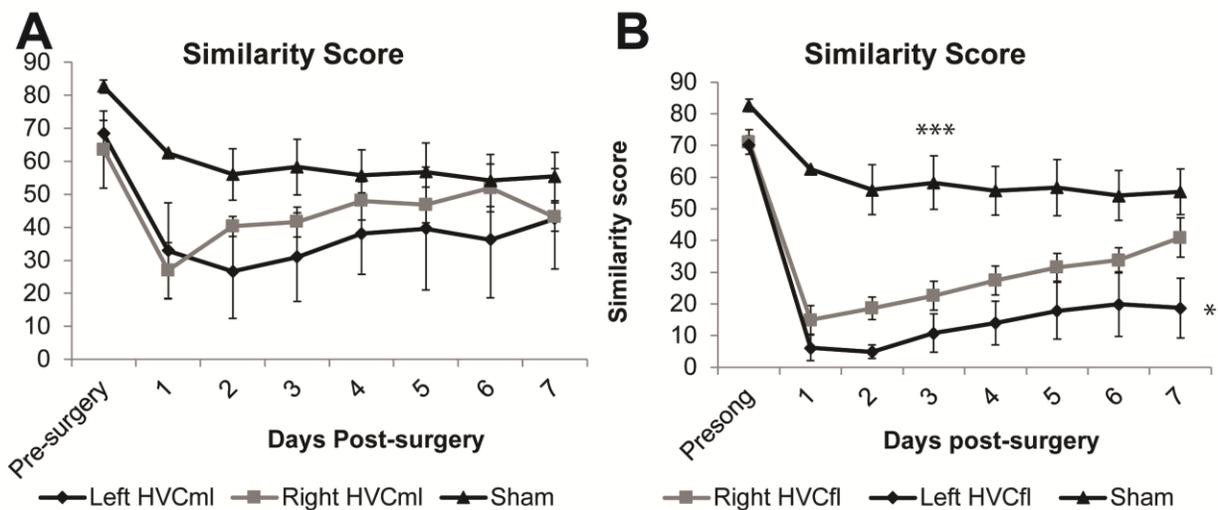


Figure 11. Rate of song recovery for microlesion and full lesion experiments. A) Similarity scores declined for sham and microlesion conditions and do not show differences in rate of recovery over one week of recording. **B)** Similarity scores declined significantly more for full lesion conditions than sham animals, $*** p < 0.005$. Right full lesion animals show a faster rate of recovery than left lesion animals, $* p < 0.05$.

Higher frequency syllables return more quickly after right HVC full lesion

Right lesion males show evidence of reincorporating higher frequency syllables by PSM 5, whereas proportions in the left HVC lesion songs remain static across time (Table 4). In the left lesion group, two males rarely produced higher frequency syllables (e.g., BFW50 and BFR100, see Appendix A).

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Table 4. Percent increase and decrease in syllable type at PSM1 and PSM5

		PSM1	PSM5
Right HVC lesion	Lower frequency	29.56% ± 16.09	29.53% ± 15.63
	Higher frequency	-68.99% ± 7.59	-29.28% ± 24.03
Left HVC lesion	Lower frequency	22.92% ± 12.26	22.31% ± 10.56
	Higher frequency	-57.89% ± 20.91	-66.55% ± 20.88

Between-groups comparisons at post-surgery month 1

Spectral and temporal features of HVC-lesioned song largely recover by post-surgery month 1. A one-way ANOVA of the average song similarity score was not significantly different across sham, left full lesion and right full lesion groups ($F(2,14) = 2.768$, $p = 0.103$, Figure 12A).

Decline in peak frequency persists after 1 month of recovery. A one-way ANOVA for peak frequency resulted in significant between-subjects differences ($F(2,17) = 14.216$, $p < 0.001$, Figure 12B). The peak frequency of sham animals ($10.87\% \pm 0.05$) was significantly higher than both lesion conditions. Right full lesion animals ($-19.72\% \pm 2.98$) were also significantly higher than left lesions ($-37.45\% \pm 9.77$).

Amplitude decline is sustained after left HVC damage. A one-way ANOVA for amplitude at PSM1 resulted in significant between-groups difference ($F(2,17) = 4.031$, $p = 0.04$, Figure 12C). Sham ($-7.89\% \pm 6.73$) and right lesion ($-5.64\% \pm 9.27$) were both significantly higher than left lesion ($-39.28\% \pm 11.81$).

Songs remain “noisier” at post-surgery month 1. A one-way ANOVA for entropy resulted in significant between-groups differences ($F(2,17) = 7.024$, $p = 0.007$, figure 12D). Mean entropy of sham animals ($-3.69\% \pm 2.48$) was significantly lower than

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both lesion conditions. Left ($19.47\% \pm 6.36$) and right ($14.11\% \pm 5.36$) lesion conditions were not different from each other ($p = 0.46$).

Left HVC-lesioned song inserts abnormally long song syllables. A one-way ANOVA for syllable duration resulted in significant between-groups differences ($F(2,17) = 5.646$, $p = 0.015$, Figure 12E). Syllable duration of sham ($1.89\% \pm 3.73$) and right lesioned ($-6.18\% \pm 5.68$) animals was significantly shorter than the left lesioned group ($56.53\% \pm 4.59$). At post-surgery month 1, syllables are produced with greater stability from one bout to the next and individual variance is much lower compared to the first week. Thus, we can reliably report that abnormal long syllables are either “noisy” broadband stacks or harmonic syllables with a fundamental frequency below 2.2 kHz.

Long silent gaps remain in post-surgery month 1 song. A one-way ANOVA for ISI resulted in significant between-groups differences ($F(2,17) = 7.231$, $p = 0.006$, Figure 12F). The ISI of sham animals ($-1.06\% \pm 1.06$) were significantly shorter than both left ($39.91\% \pm 9.12$) and right ($32.09\% \pm 11.64$) HVC-lesioned animals. There were no differences between left and right lesion groups.

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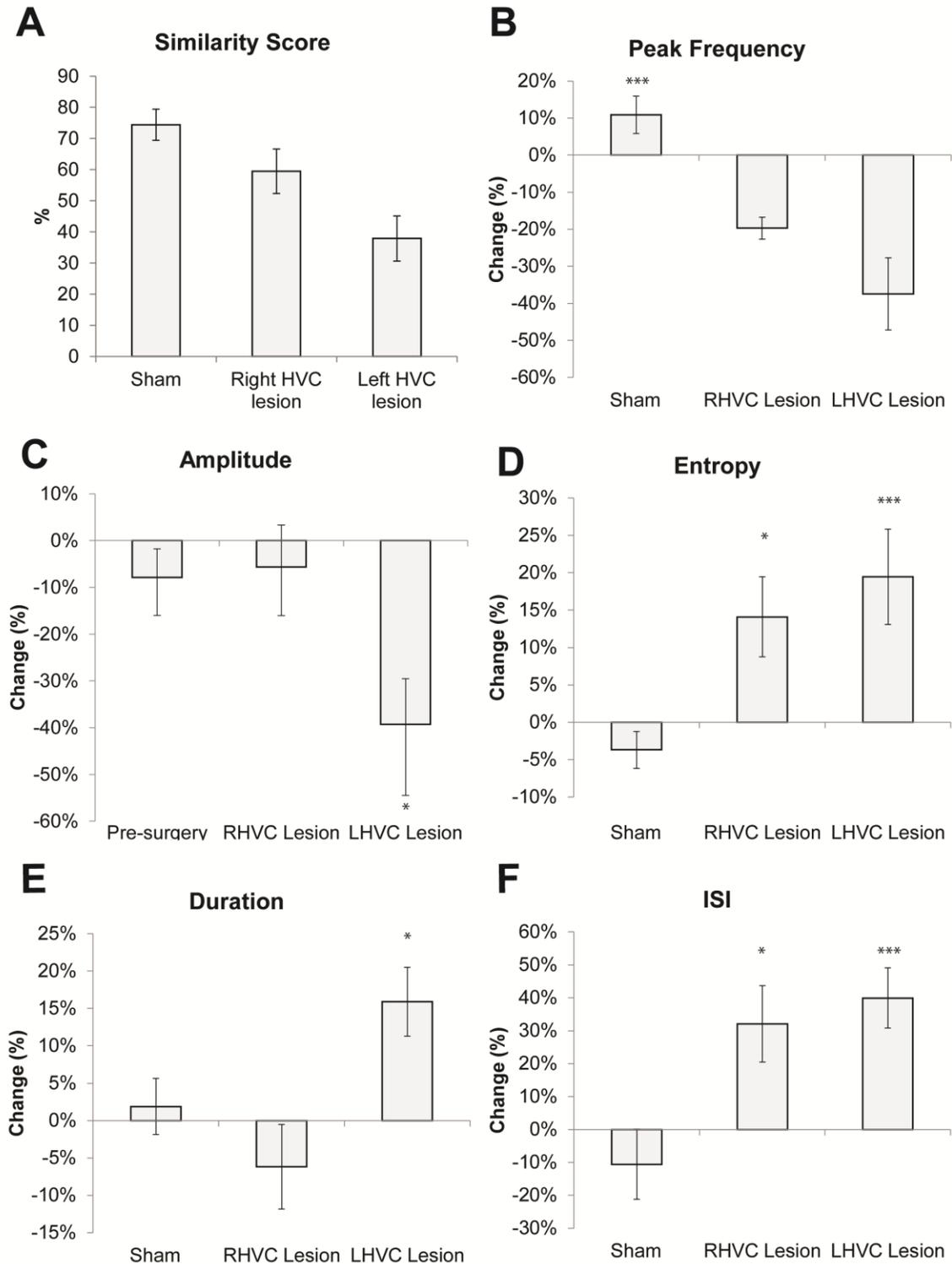


Figure 12. Sham, left and right HVC lesion comparisons at post-surgery month 1. Group means \pm SEM for a) similarity score, b) peak frequency, c) amplitude, d) entropy, e) duration, and f) ISI. Left full lesions are significantly different from shams in all measures except similarity score. Mean duration and amplitude of right HVC lesion condition are similar to shams. * $p < 0.05$, * $p < 0.005$.**

Peak frequency remains lower in left lesion condition at post-surgery month 5.

Similarity score. A significant difference between left and right HVC lesion was not observed ($t(6) = 4.246$, $p = 0.259$, Figure 13A).

Peak frequency. There was a significant effect of lesion side for peak frequency ($t(6) = 1.265$, $p = 2.737$, $p = 0.034$, Figure 13B). Mean peak frequency of the left lesion condition was significantly lower.

Entropy. A significant difference between left and right HVC lesion was not observed ($t(6) = -0.518$, $p = 0.623$, Figure 13C).

Amplitude. A Levene's test indicated unequal variances ($F = 8.758$, $p = 0.02$), therefore, degrees of freedom were adjusted from 6 to 4.295. A significant difference between left and right HVC lesion was not observed ($t(4.295) = -0.730$, $p = 0.493$, Figure 13D).

Duration. A significant difference between left and right HVC lesion was not observed ($t(6) = -0.471$, $p = 0.654$, Figure 13E).

Inter-syllable Interval. A significant difference between left and right HVC lesion was not observed ($t(6) = -0.842$, $p = 0.432$, Figure 13F).

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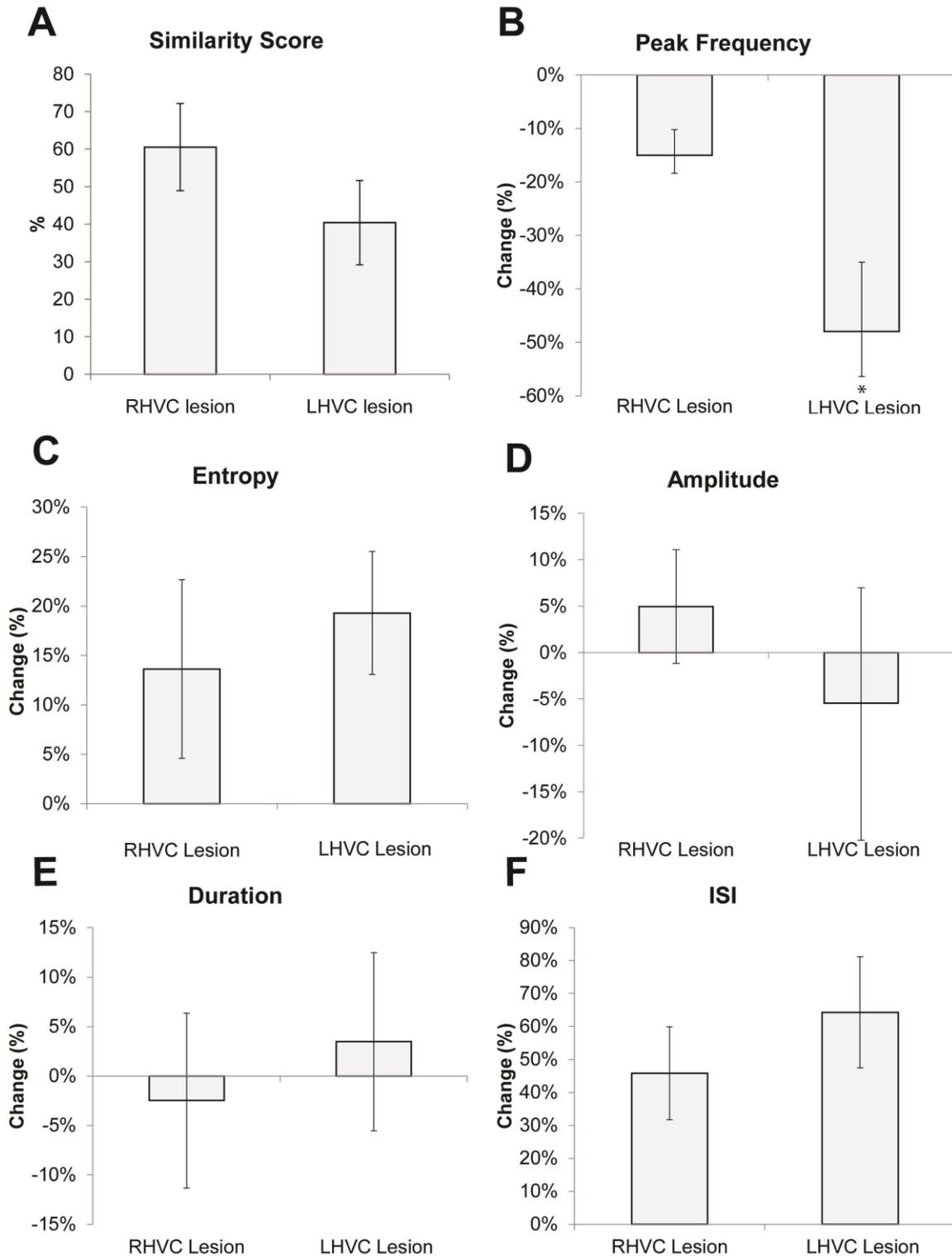


Figure 13. Left and right HVC lesion comparisons at post-surgery month 5. . Group means \pm SEM for a) similarity score, b) peak frequency, c) amplitude, d) entropy, e) duration, and f) ISI. Left HVC lesion peak frequency is significantly lower than in right lesion condition. * $p < 0.05$.

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Changes in song air sac pressure after left or right HVC lesion

Air sac pressure reflects the combined outcome of syringeal gating of airflow and active pressure generation driven by activation of the respiratory muscles. The air sac pressure pattern is a highly stereotyped motor pattern like song. We did not find differences between left and right HVC lesion groups (see Appendix D), therefore the lesion group data were collapsed in all analyses.

Expiratory air sac pressure (EP) duration. A repeated-measures ANOVA comparing EP duration across pre-surgery song, post-surgery, and 6-8 m post-surgery resulted in significant within-subjects differences ($F(2,10) = 4.528$, $p = 0.04$, Figure 14A). Pre-surgery syllable EP duration (0.10 ± 0.01) was significantly shorter than both post-surgery timepoints. EP duration did not change between post-surgery (0.11 ± 0.01) and 6-8 m post-surgery ($0.10 \pm .008$).

Expiratory air sac pressure amplitude. A repeated-measures ANOVA comparing EP amplitude across pre-surgery song, post-surgery, and 6-8 m post-surgery was performed. Mauchly's test for sphericity indicated unequal variances across timepoints, therefore, degrees of freedom and significance values were adjusted accordingly. There were significant within-subjects differences ($F(1.060,5.298) = 10.197$, $p = 0.022$, Figure 14B). Pre-surgery syllable EP amplitude (9.71 ± 1.03) was significantly greater than both post-surgery timepoints. There were no differences between post-surgery (6.44 ± 0.71) and 6-8 m post-surgery (6.80 ± 0.10) EP amplitudes.

Expiratory air sac pressure slope. A repeated-measures ANOVA comparing EP duration across pre-surgery song, post-surgery, and 6-8 m post-surgery resulted in

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significant within-subjects differences ($F(2,10) = 14.112$, $p = 0.001$, Figure 14C). Pre-surgery syllable EP slope (14.33 ± 2.01) was significantly sharper than both post-surgery timepoints. There were no differences between post-surgery (6.68 ± 1.65) and 6-8 m post-surgery (9.42 ± 2.20) EP slopes.

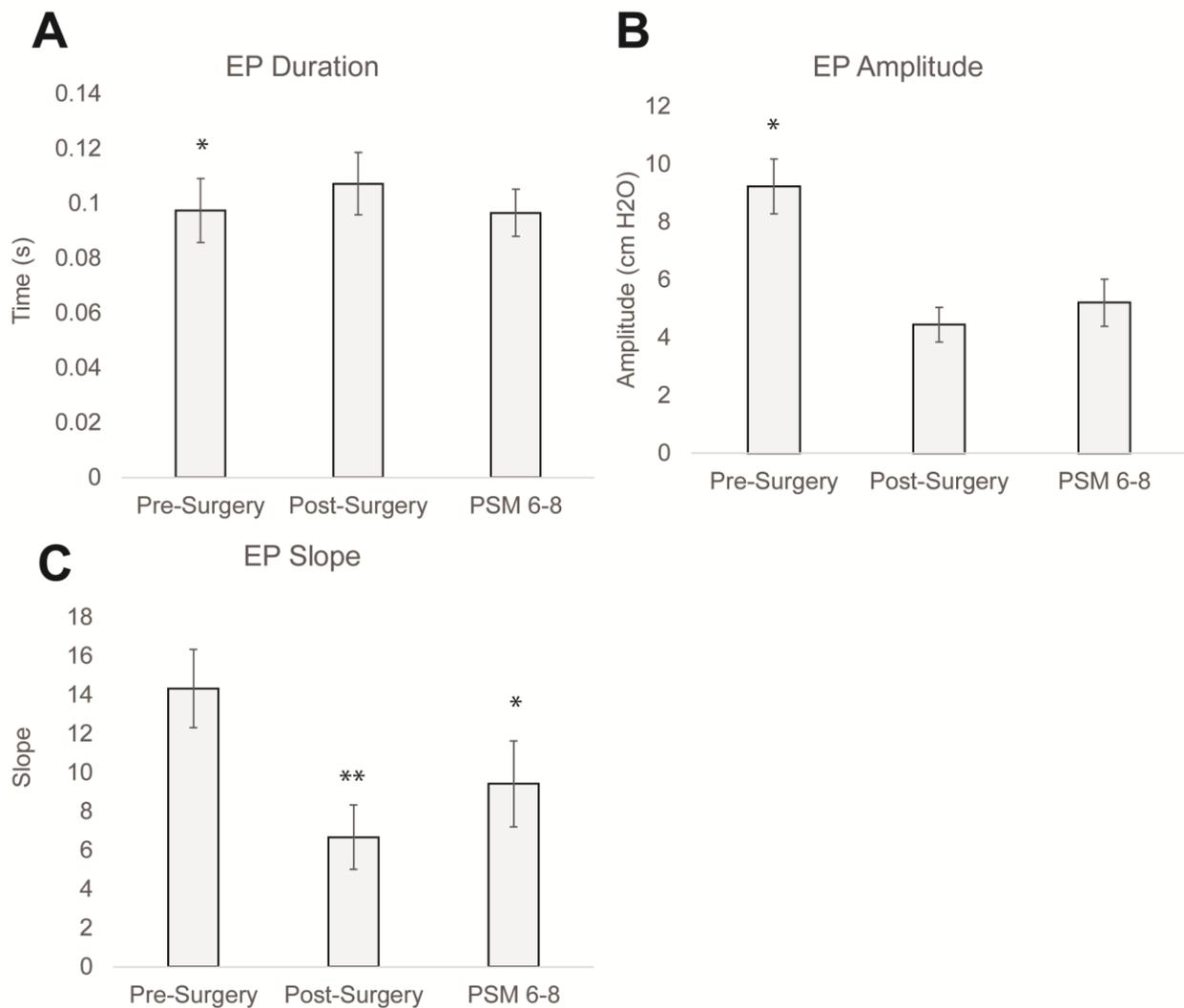


Figure 14. Group means of expiratory pulse features. Bar graphs of three recording timepoints – pre-surgery, post-surgery and 6-8 mo after surgery for a) EP duration, b) EP amplitude, and c) EP slope. Pre-surgery timepoint is significantly higher than both post-lesion conditions. * $p < 0.05$, ** $p < 0.01$.

Inspiratory air sac pressure (IP) duration. A repeated-measures ANOVA comparing IP duration across pre-surgery song, post-surgery, and 6-8 mos post-surgery

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did not result in significant within-subjects differences ($F(2,10) = 0.936$, $p = 0.424$, Figure 15A).

Inspiratory air sac pressure amplitude. A repeated-measures ANOVA comparing IP amplitude across pre-surgery song, post-surgery, and 6-8 mos post-surgery resulted in significant within-subjects differences ($F(2,10) = 7.078$, $p = 0.012$, Figure 15B). Pre-surgery syllable IP amplitude (-2.56 ± 0.31) was significantly greater than post-surgery IP amplitude ($p = 0.014$) and marginally greater than 6-8 mos post-surgery IP amplitude ($p = 0.065$). There were no differences between post-surgery (-1.53 ± 0.24) and 6-8 mos post-surgery (-1.94 ± 0.39) IP amplitude.

Inspiratory air sac pressure slope. A repeated-measures ANOVA comparing IP slope across pre-surgery song, post-surgery, and 6-8 m post-surgery was marginally significant ($F(2,10) = 3.541$, $p = 0.069$, Figure 15C). Pre-surgery syllable IP slope (-4.48 ± 0.60) was marginally sharper than post-surgery IP slope ($p = 0.066$) and was not different from 6-8 m post-surgery IP slope ($p = 0.134$). There were no differences between post-surgery (-3.20 ± 0.62) and 6-8 m post-surgery (-3.86 ± 0.13) IP slope.

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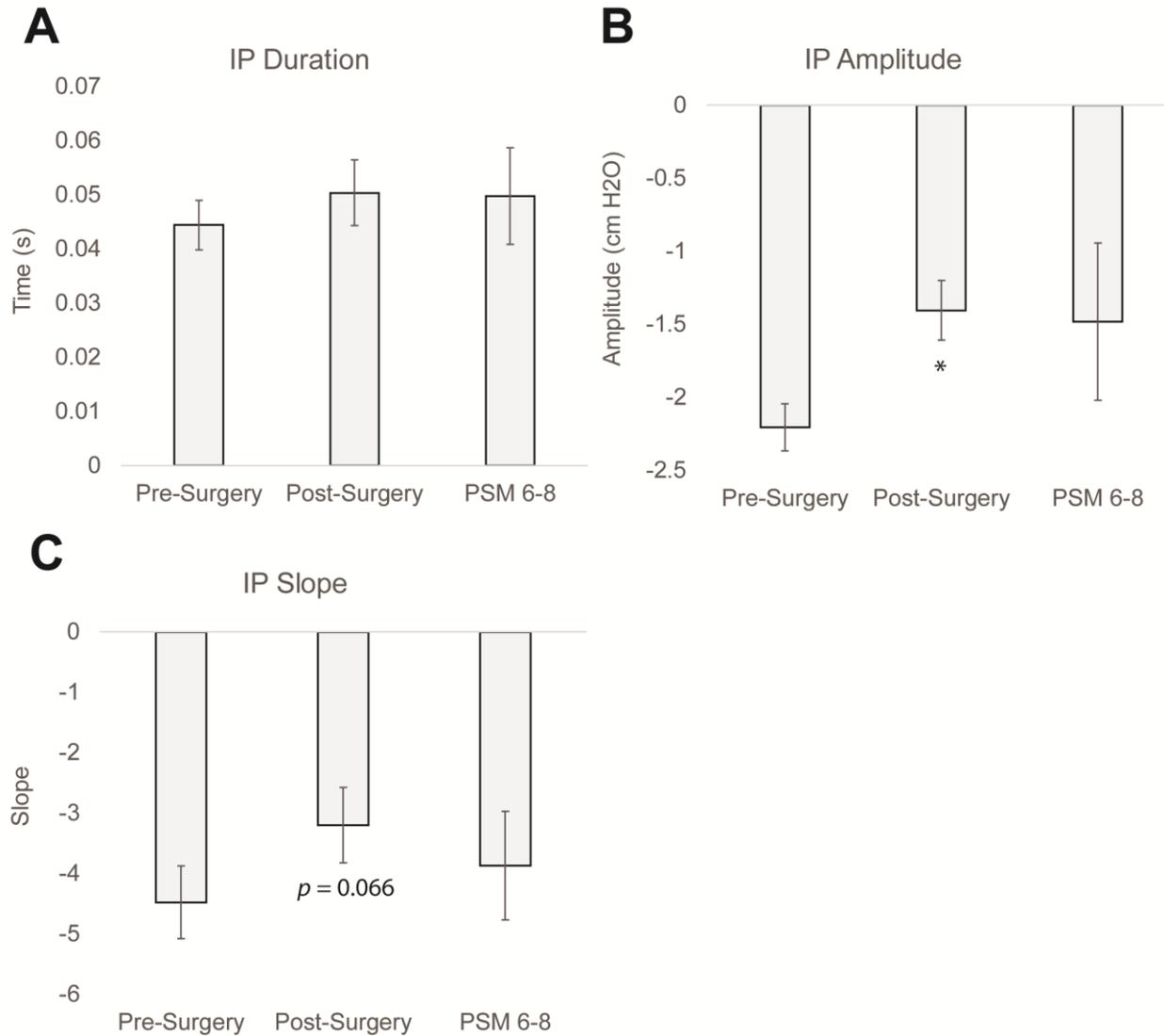


Figure 15. Group means of inspiratory pulse features. Bar graphs of three recording timepoints – pre-surgery, post-surgery and 6-8 mo after surgery for a) IP duration, b) IP amplitude, and c) IP slope. * $p < 0.05$.

Changes in contact/distance call air pressure after left or right HVC lesion

A comparison of pre- and post-surgery contact calls did not change EP duration, amplitude, or slope ($p > 0.1$, Figure 16).

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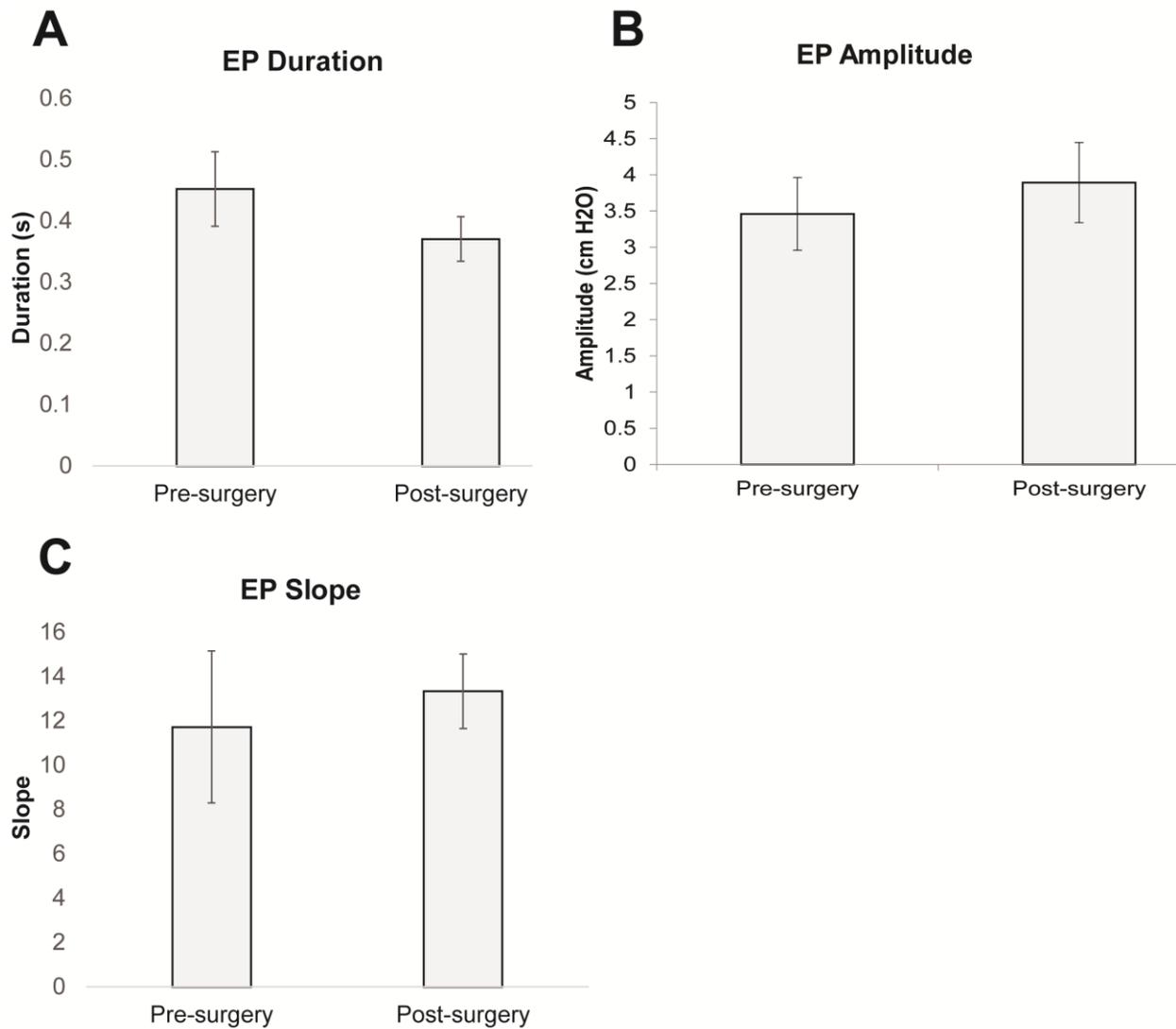


Figure 16. Respiratory features of contact calls are unaffected by HVC lesion. Group means \pm SEM of a) EP duration, b) EP amplitude and c) EP slope.

Summary and Discussion

Evidence of hemispheric dominance for peak frequency, song memory and recovery

Contrary to our expectations, left and right microlesion conditions followed a similar pattern of recovery (Figure 11A). Further, there was a similar decline in song similarity in the sham lesion condition. As such, it is possible that microlesions are insufficient for evaluating the effects of left and right HVC damage on song recovery. However, we did find evidence for left hemisphere dominance in the full lesion animals.

Similarity scores illustrate that after damage to left HVC, song does not recover by PSD7. In contrast, the right lesion condition shows moderate recovery and is comparable to the sham condition (Figure 11B). At PSM1, within-group variability is lower in both lesion conditions, indicating songs are stably produced within and across individuals. At PSM1, Left HVC lesion animals are different from the other conditions in three song features: syllable peak frequency, amplitude and duration. Left lesion animals were still singing songs that were characterized by soft, predominately lower frequency, and abnormally long duration, novel syllables.

At PSM 5, songs in the left lesion group remained altered compared to the presurgery song. Two out of four males sang broadband, lower frequency stacks. Although all experimental animals sang fewer higher frequency syllables, the average peak frequency in the left lesion condition remained significantly lower than the right lesion condition (Figure 13B).

Addressing whether the memory of higher frequency syllables or the song itself is stored in left HVC or its afferents is outside the scope of this study. Nevertheless, these

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results corroborate previously published findings on left-hemisphere dominance in the Bengalese finch (e.g. Okanoya, Ikebuchi, Uno, & Watanabe, 2001; Okanoya, 2004) and provide evidence that suggests left HVC is necessary for producing higher frequency syllables of species-typical acoustic and temporal properties.

Impaired respiratory motor pattern influences persistent acoustic and temporal deficits

As mentioned in chapter 2, all experimental animals showed significant changes in temporal song features. Silent intervals between syllables were longer. Duration, initially highly variable, and stabilized into an altered state (Figure 12E). These features suggested that HVC damage impaired the underlying song motor pattern. Syringeal and respiratory electromyography in the brown thrasher have shown that respiration is bilaterally controlled in the intact animals (Goller & Suthers, 1999). Studies by Aronov and colleagues have suggested that bilateral HVC lesions in the zebra finch disrupt the song rhythm by eliminating stereotyped syllable and silent gap duration (Veit et al., 2011; Aronov et al., 2008, 2011). Bilateral HVC lesions in the adult canary, however, has shown that song may not be identifiable after lesions (Nottebohm, 1976). By performing unilateral lesions, we 1) are able to identify song, 2) replicate and extend those findings by documenting changes in respiratory features, and 3) demonstrate the absence of lateralized HVC contribution to respiratory features.

After HVC lesion, the amplitude and rate of pressurization (slope) of song EPs are significantly lower. This finding explains the amplitude and temporal changes observed in chapter 2. Lower air sac pressure will translate to lower phonatory airflow,

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leading to softer song. This fact alone points towards disrupted motor control of expiratory muscles.

Slower rate of pressurization indicates that HVC lesions impair the respiratory-syringeal motor system, but there are three factors that can be affected: 1) vocal tissue adductors that are responsible for gating airflow during song, 2) expiratory muscle activation that generates the airflow, and 3) or a decoupling of these two highly synchronized motor systems. In chapter 4, we address which of these factors contribute to the respiratory and acoustic changes. What is currently clear is that a slower rate of pressurization explains the abnormal ISI and syllable duration.

Veit and colleagues (2011) have documented that juveniles singing subsong will produce EPs that are longer duration than its syllables. There will either be a silent gap at the beginning or the end of the EP, suggesting that a key characteristic of poor respiratory-motor coordination is the inability to phonate for the entire expiration. Our results in adult Bengalese finches support this notion; a slower rate of pressurization can directly impact a bird's ability to produce their rapidly modulated song, which leads to temporal and acoustic distortion.

Acoustic features recover more quickly than respiratory features

It is of note that while acoustic features show a steady pattern of recovery months after lesion, EP amplitude, slope, and duration remained significantly lower than baseline measures at PSM 6-8. IP features were less affected by HVC lesion: duration exhibited no differences between pre and post-surgery timepoints, slope showed strong evidence of recovery. IP amplitude followed the same pattern of decline and minimal recovery as EP features. These results suggest that persistent peak frequency, syllable

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amplitude and ISI changes at PSM 5 are directly affected by sustained respiratory motor deficits.

HVC lesions do not impair the respiratory pattern of learned contact calls

We replicated our previous findings that HVC lesions do not affect contact calls. Just as acoustic and temporal features are not altered by HVC damage, we show that respiratory features that underlie contact calls remain intact. Although contact calls are learned vocalizations, they are not produced with a particular syntactic order. Thus, HVC appears to importantly contribute toward the production of sequential, learned vocalizations.

Chapter 4: Reduced HVC input impairs amplitude and temporal synchronicity of song-related respiratory muscle activity

These final two studies explore why HVC damage abolished stereotyped song respiratory patterns. Thus far, we have established a strong tie between left HVC and the biomechanical properties of the left sound source. Following extensive damage to HVC, the left side is more critical than the right for reacquisition of higher frequency song syllables. We have also demonstrated that unilateral HVC damage produces temporal abnormalities and quieter songs by disrupting respiratory motor control. With the exception of peak frequency after left HVC lesion, acoustic features of song recover by six months post-lesion in full lesion animals. However, song-related air pressure patterns do not recover by six to eight months post lesion. Thus, both sides of HVC are equally important for the generating normal singing air pressure.

The time-locked relationship between HVC and the production of song syllables is well established. Multiunit and single-unit recordings show that HVC neurons produce stereotyped bursts of activity in singing birds and are highly responsive to playback of the bird's own song (Yu & Margoliash, 1996; Schmidt, 2003; Kozhevnikov et al., 2007; Nick & Konishi, 2005a). It is agreed upon that the HVC to RA pathway is the likely key to producing adult, stereotyped song. Song remains well-conserved if the LMAN to RA pathway is obstructed by lesion, even if auditory feedback is impaired by deafening or if HVC output is manipulated with microlesions (Brainard & Doupe, 2000; Frank & Johnson, 2007). Ablation of Area X-projecting HVC neurons does not affect song

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production (Scharff, et al., 2000). These studies emphasize that song will remain intact provided HVC continues to send signals downstream.

As of now, the information relayed by HVC-RA neurons is unknown. Relative to other classes of HVC neurons, HVC-RA neurons rarely fire and are difficult to isolate. Using antidromic stimulation, Fee and colleagues were able to identify HVC-RA projection neurons and record activity in sleeping and awake zebra finches. A single HVC-RA neuron will burst once during a song motif and although it is presumed that this burst is followed by activity in RA, this has yet to be demonstrated in singing animals. In sleeping birds, they report one-third of recorded HVC and RA neuron pairs show sequential firing patterns and there is evidence to suggest song-specific neural activity is being rehearsed in the sleep-state (Hahnloser, Kozhevnikov & Fee, 2002; Dave & Margoliash, 2000). Fee and colleagues have recorded over 100 (possibly 186 across three publications) HVC-RA neurons and have concluded these projection neurons fire in a time-locked, song-specific manner, but are not tied to any particular acoustic or temporal feature, such as the onset or offset of syllables (Hahnloser, Kozhevnikov & Fee, 2002). However, they found that the majority (61%) of HVC interneuron bursts are synchronized with HVC-RA bursts. As interneurons are highly active, they have argued that one could make inferences of HVC-RA firing patterns based on pairs of HVC interneuron and RA neurons. They found that 58% of RA bursts is either preceded by (<10ms) or overlaps HVC interneuron activity and thus, each RA burst is very likely driven by HVC-RA activity (Fee, Kozhevnikov & Hahnloser, 2004). However, given that an interneuron can spike anywhere from 20 to 208 times within a song motif, this is not

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sufficient evidence for measuring interneuron activity as a predictor of HVC-RA projection neuron activity (Kozhevnikov & Fee, 2006).

In 2013, Amador and colleagues presented indirect evidence that HVC-RA neurons are tied to vocal motor “gestures” that shape the respiratory-syringeal-oro-esophageal system. They developed a complex mathematical model of the biomechanics of oscine vocal production that accounts for expiratory airflow, vocal tissue adduction and resistance, and upper vocal tract filtering. Using this model, they created a synthetic variant of a bird’s song simplified to modulations in pressure and vocal tissue (labial) tension. This synthesized song was played back to the bird during sleep and they recorded HVC-RA and HVC interneuron activity. Unlike the studies from Fee and colleagues, Amador et al., (2013) attempted to break down the song into respiratory units and look at the temporal relationship between the firing patterns of HVC neurons and the underlying respiratory motor pattern. They argue that HVC-RA neurons fired at points that necessitate a change in the respiratory-syringeal-oro-esophageal system. These points coincide with rapid frequency shifts, onset and offset of syllables, and the transition from harmonic to chaotic sound. They also reported that bursts were more likely to occur during changes in pressure, rather than labial tension. Lastly, they observed that the minima of interneuron bursting is time-locked to gestures, suggesting that within 10 ms of a vocal motor gesture, interneurons are briefly suppressed to permit excitatory HVC activity. What the GTE model would suggest is that the local HVC circuitry is responsible for the sparse projection neuron bursting. Normally, interneurons are tonic and highly active except at time-locked instances wherein HVC sends a signal to RA and/or Area X.

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Despite the lack of direct evidence, alternative methods of determining whether HVC regulates the temporal features of song have been extremely informative. Fee and colleagues have created a miniature Peltier device with gold plates bilaterally situated over HVC. Capitalizing on the fact that HVC is 200-700 μm ventral to the surface, with the Peltier device, they could manipulate the temperature of local HVC activity without disrupting the song system. These studies have established cooling HVC in zebra finches “stretches” song motifs by 1-3 percent per degree Celsius (Long & Fee, 2008; Andalman, et al., 2011). Focally cooling RA (with gold pins as RA is 2-3 mm ventral) produces a slight temperature change in HVC (approximately 30 percent of RA’s temperature change) and a slight stretch. However, once Long and Fee (2008) controlled for the change in HVC temperature, they found that RA cooling was not sufficient to alter temporal dynamics of song. A replication with simultaneous air sac pressure recording demonstrated that the song motif “stretch” effect is produced by elongated expiratory pressure pulses (Andalman, et al., 2011). They provide evidence that HVC input is closely tied to the onsets and offsets of an EP, and by extension, the song syllable.

Altogether, these studies paint a picture wherein HVC activity must always precede a song motif or a song syllable. The level of precision is currently unknown. What is also unknown is “why”. Is HVC projection neuron activity independent of the syllables produced and the rate at which they are sung? In which case, HVC’s sequential bursts act as a clock or a rhythm generator. Or are the output of HVC projection neurons directly involved song, making the magnitude and timing of these sparse bursts important?

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To address this question, we briefly discuss the biomechanics of phonation. Phonation occurs when vocal tissue vibrate and the mechanical oscillations transform air into sound. Human and oscine vocal tissue are part of a myoelastic-aerodynamic system with key features: 1) vocal tissue are voluntarily adducted into the bronchial/tracheal lumen, 2) phonation is initiated by pressure levels under the vocal tissue that are high enough to force air past the vocal tissue, and 3) a pressure differential exists above and below the vocal tissue that creates a Bernoulli effect to sustain the push and pull of vocal tissue (Titze, 1988; Riede & Goller, 2010).

Direct evaluation of the relationship between muscle activation, subglottal pressure and sound is currently impossible in humans, however, this system is the foundation for speech and one of the first complex skills infants will master. Human literature is limited to minimally invasive methods of evaluation (e.g. endoscopic observation, *ex vivo* tissue preparation, plethysmography, or surface EMG) and mathematical modeling, although a direct comparison of subglottal and intraoral pressure in one subject has been published (Hertegard, Gauffin & Lindestad, 1995).

In songbirds, syringeal and respiratory muscle activity has been recorded in awake and singing animals. Expiratory muscle (*m. obliquus externus abdominis*) activity is time-locked to expirations and higher levels of activity occur with vocal production (Goller & Suthers, 1999). These muscles provide the pressure head that sets the adducted vocal tissue into oscillation. Electromyogram recording of the principal syringeal adductor, dorsal tracheobronchialis muscles (dTB), demonstrates that muscle activation is greatest during the rise of subsyringeal air pressure and muscle activity decreases at syllable onset (Goller & Suthers, 1996; Vicario, 1991). Thus, the typical

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pattern of events during vocalization is dTB activation to adduct the vocal tissue followed by expiratory muscle contraction to increase subsyringeal pressure, and modified reduction of dTB activity to permit airflow.

What we observed in Chapter 3 is that unilateral HVC lesion disrupts song production in some fashion. As subsyringeal air pressure (which was our measure for the respiratory motor pattern) is affected by both syringeal and expiratory muscle activation, our goal was to determine how the reduction of HVC output translates into disruption of air pressure generation. I manipulated HVC output via sequential lesion or transient inactivation, and recorded from expiratory muscles or performed simultaneous expiratory and dTB electromyogram recordings. I predict that there will either be a decoupling in respiratory and syringeal muscle activity and/or an overall reduction in muscle activation following removal of left or right HVC.

Methods

Subjects

14 male Bengalese finches were used in these two experiments (Experiment 4: n = 5; Experiment 5: n = 9). Finches were purchased from local suppliers.

Housing Conditions

Birds were housed in communal flight cages until the start of the experiment, and kept on a 14:10 light:dark cycle during housing and experimental testing. They were fed seed *ad libitum*, and their diet was supplemented with vitamins vegetables and egg. During data collection, birds were kept in individual cages (31.8×10.5×25.4 cm) and the microphone was placed 14 centimeters centered above the perch.

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Surgical Overview and Timeline

Experiment 4: Sequential HVC Lesion. Multiple sequential surgeries were performed. First bilateral lesion electrodes were permanently implanted into each HVC. The electrode implant consisted of an insect pin (#00, 0.10 mm) soldered to a gold pin, which was permanently fixed to the birds skull with dental cement. After birds recovered from surgery, and habituated to carrying the weight of the backpack for recording pressure and electromyogram activity, a cannula was inserted into a posterior thoracic air sac, and expiratory EMG electrodes were inserted into the abdominal muscles. Baseline song was collected for 1-2 days. Once baseline song was collected, a unilateral HVC lesion was performed by passing 100 μ A of current for 90 s. Birds were recorded for 3-4 days. In a subset of animals with usable air sac pressure, we lesioned the contralateral HVC and collected song for an additional 3-4 days.

Experiment 5: Unilateral HVC Inactivation. Custom-built microdialysis probes were built with a reservoir length of 50 mm (ID: 0.254 mm, OD: 0.3048 mm) with 500 μ m of dialysis membrane (ID: 200 μ m, OD: 216 μ m, diffusion rate: 3ml/h, MWCO 13 kD, SpectrumLabs, Rancho Dominguez, CA) exposed at the tip. Day 1, we inserted expiratory and syringeal EMG electrodes. Day 2, we implanted bilateral custom-built microdialysis probes in HVC. The dialysis probes were fixed to the skull with dental cement. Once the birds recovered (1-2 days), we inserted the air sac pressure cannula and collected baseline song. After baseline song was collected, we transiently inhibited either left or right HVC activity by infusing muscimol, a gamma-Aminobutyric acid A (GABA_A) agonist for 4-6 hours using constant diffusion through the dialysis membrane.

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Before and after four to six hour experimental session, the microdialysis probes were filled with sterile saline.

Pre- And Post-Surgical Procedures

For all surgeries, birds were deeply anesthetized by vaporizing isoflurane (1-2%) in breathing air (flow rate, 2 L/mg). Surgical anesthesia was confirmed by an absence of physical movement following a light pinch on one of the bird's talons. Birds recovering from surgery were kept in individual cages and monitored until they perched.

Surgical Procedures

Electrode Implant. Feathers were removed to expose the ears and scalp. Anesthetized birds were placed on a stereotaxic frame and secured with ear bars. An incision was made along the anterior to posterior axis, parting the skin and exposing the skull. Using the mid-sagittal sinus as the point of origin, we used the distinctive electrophysiological activity of HVC neurons to identify the mediolateral and dorsoventral boundaries of the nucleus. We used a differential AC amplifier (5.0 kHz low-pass, 100 Hz high-pass, 10 kHz gain; 1700 A-M Systems, Sequim, WA) and a 3 inch diameter tungsten, parylene-c insulated electrode to record multiunit neural activity and identify the boundaries of HVC (Microprobes, Gaithersburg, MD). Custom-built miniature electrodes were lowered to a depth of 500 μm . Microdialysis implants were lowered to a depth of 900 μm to ensure the exposed membrane was within the HVC nucleus. Implants were sealed in place with dental cement.

Air Sac Cannulation. Anesthetized birds will be placed in a supine position. A small opening will be made just below the rib cage into a thoracic air sac. A 6.5 mm length of silastic tubing (0.76 mm I.D.; Dow Corning, Midland, MI) will be inserted into a

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posterior thoracic air sac and sutured to the third rib to minimize cannula movement.

The free end of the tube will be attached to a custom-built piezo-resistive pressure transducer (Fujikura, FPM 02PG; Santa Clara, CA) that will be attached to a Velcro tab centered on the bird's back.

Electromyogram Electrode Insertion

Expiratory Muscle Recording. Anesthetized birds were placed on their side with the ipsilateral wing extended behind them and the ipsilateral leg flexed in the cranial direction to expose the expiratory abdominal muscle normally obscured by the femur. A 1 cm incision was made perpendicular to the arch of the pelvis extending to the rib cage. A pair of stainless steel electrodes (0.001"; California Fine Wire, Grover Beach, CA) were braided and inserted into the expiratory muscle (*m. rectus abdominis*). The wires were routed under the skin towards the base of the neck and attached with silver epoxy (MG Chemicals, Surrey, B.C., Canada) to 36 gauge wires mounted on a backpack carried by the birds.

Syringeal Muscle Recording. Feathers were removed from the ventral surface of anesthetized birds and the thoracic cavity will be opened. A small incision in the intraclavicular air sac was made with fine micro-dissecting scissors and pairs of braided electrodes (0.001 in; California Fine Wire, Grover Beach, CA) were inserted into either the left or right half of the exposed dTB. The air sac was sealed with tissue adhesive, and the electrode wires were sutured to the clavicle and routed under the skin to the base of the neck. The free end of the wires were attached to 36 gauge wires with silver epoxy, and the wires were mounted on a pressure transducer and backpack assembly carried by the birds.

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Data Acquisition

Microphone and physiological measurements were recorded simultaneously and saved as a multi-channel .wav file (1 to 5 channels). The microphone signal was amplified and high-pass filtered (300 Hz; RME audio, Haimhausen, Germany). Air sac pressure signal was amplified 100x and low-pass filtered (6 kHz cutoff, Brownlee Precision, Model 440, Santa Clara, CA). EMG signals was amplified 1000x and band-pass filtered (100 Hz high-pass, 5.0 kHz low-pass; Brownlee Precision, Model 440, Santa Clara, CA). All recordings were digitized (22.05 kHz sample rate, 16 bit resolution) via an analog-to-digital converter (National Instruments, NI USB-6251, Austin, TX), and saved to disk on a computer running Avisoft Recorder software whenever the microphone or pressure signal exceeded a user-defined threshold for longer than 10 ms (Avisoft Recorder, Avisoft Bioacoustics, Berlin, Germany).

Data Analysis

Air Sac Pressure Analysis. Using custom-written LabVIEW (National Instruments, Austin, TX) software, expiratory pulses (EPs) and inspiratory pulses (IPs) were segmented based on a user defined estimate of ambient pressure. The EPs and IPs produced during song were identified using a custom written program, and the mean amplitude, pulse slope, and duration were measured. The voltage output range of the pressure transducers were calibrated to cm H₂O using a manometer (see Chapter 3). Thus, all pressure measurements are reported as cm H₂O.

Electromyogram Analysis. Using custom-written LabVIEW software, we will segment expiratory EMG activity produced during song. We defined the onset of the EMG signal 15 ms before the start of the expiratory air sac pressure pulse. Segmented

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EMG signals were rectified and averaged with a 1 ms smoothing window. We measured mean and peak amplitude over the entire waveform, relative to the onset of the expiratory pressure pulse. As EMG activity is highly variable across bouts, amplitude was normalized as a fold increase compared to activity during quiet inspiration. Fine temporal changes as a result of losing synchronization between respiratory and syringeal motor systems was measured in 1) the initial rising slope of muscle activation occurring during the first 5-20 ms of the muscle activity or 2) changes in the interval (ms) between the onset of dTB and EXP activity (Figure 17).

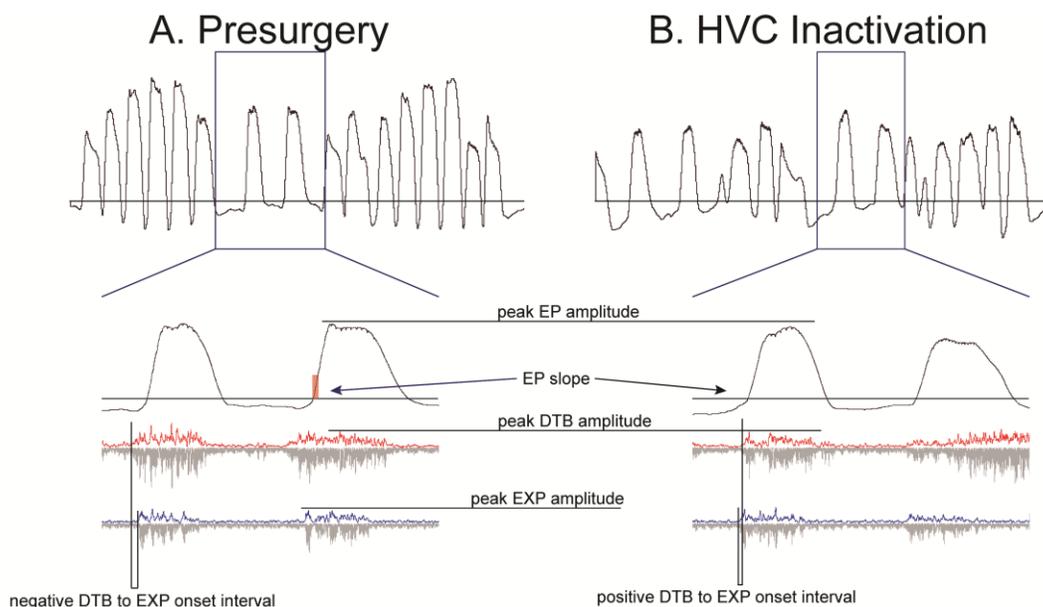


Figure 17. dTB to EXP onset interval. The interval between DTB onset and EXP onset was visually identified and measured. In majority of presong syllables, DTB activation (*red trace*) preceded EXP (*blue trace*) activity (negative interval value). However, during HVC inactivation, DTB often occurred after activation of EXP muscles (positive interval value).

EMG Cross-correlation. We also explored in whether temporal activity patterns between dTB and EXP muscle activation were similar. Waveforms were rectified, smoothed with a 1 ms window, and shifted ± 10 ms to identify the time with the highest correlation.

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Statistical Analysis

Repeated measures ANOVA for expiratory and inspiratory pressure changes. Group means in EP and IP features were compared across conditions using a repeated-measures ANOVA to determine changes following sequential lesions or to illustrate the decline and recovery during transient HVC inactivation.

Within-subjects t-test. The average dTB and EXP amplitude and slope of each bird were compared across pre-inactivation and inactivation conditions.

Correlation. In a sample of song syllables, we computed a linear correlation to investigate the relationship between five different muscle activation measurements (dTB and EXP peak amplitude, EMG cross-correlation, and dTB to EXP onset) and two air pressure measurements (EP pressure and slope).

K-S test. To quantify the effects of HVC inactivation on dTB and EXP coupling, we compared the two frequency distributions of onset intervals prior to and during inactivation of HVC. A sample of 106 preinactivation syllable onset intervals was compared to the onset intervals in 100 inactivation syllable onset intervals using a Kolmogorov-Smirnov test (K-S test).

Histology

After data were collected, animals were euthanized via overdose of anesthetic isoflurane. The brain was extracted and stored in formalin until further processing. Brains were embedded in Tissue-tek (Sakura Finetek USA Inc., Torrance, CA) and sectioned at 50 μm thickness.

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Unilateral and bilateral electrolytic HVC lesion. Coronal sections were stained with cresyl violet. The anterior-to-posterior extent of HVC damage was measured by counting the number of sections with HVC damage.

HVC dialysis implant. Sagittal sections were stained with cresyl violet. I confirmed the accuracy of the dialysis implant through visual inspection. An implant was considered a success if the dialysis tract remained within the boundaries of HVC.

Results

Lesion verification

Unilateral and bilateral electrolytic HVC lesion results. In the five males in this study, there was a range of 300 – 900 μm of HVC damage. Nine sections from one bilaterally lesioned male were lost, so the magnitude of damage is unknown. However, there was evidence of at least 400 μm of damaged HVC in this individual. Given our estimates of HVC encompassing about 1000 μm of tissue sections in each animal, there was a 30-90% HVC lesion extent. In addition to HVC, there was also damage in the anterior and ventral areas surrounding HVC.

HVC dialysis implant. We verified the accuracy of the probe implant in 8 of 9 animals. An example tissue section below illustrates a successful dialysis implant (Figure 18). The tract of the microdialysis probe was found in in the anterior edge of HVC in four animals and midposterior areas of HVC in four animals.

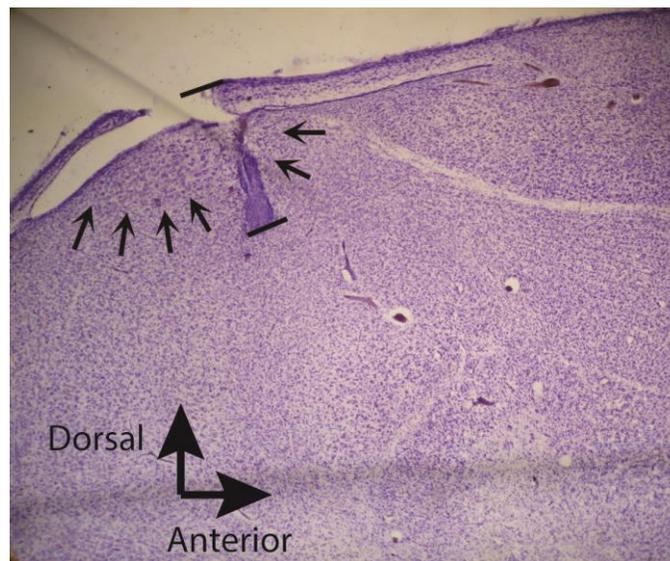


Figure 18. Sagittal section of HVC and dialysis implant. 50 μm sections were stained with cresyl violet for verifying implant accuracy. Boundaries of HVC are denoted with arrows. Depth of the implant tract is marked with a black line. This implant was on the anterior edge of HVC.

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Sequential HVC lesions

Linear decline in expiratory pressure amplitude and slope. EP amplitude decreased by $43\% \pm 0.10$ after unilateral HVC lesion ($n = 5$). Slope decreased by $64\% \pm 0.09$. Group means and individual trajectories are displayed in Figure 19. In a subset of animals ($n = 3$), air sac pressure lasted long enough to collect bilateral lesion data. There was a linear decrease in amplitude and slope after each lesion (Figure 20). A repeated-measures ANOVA for amplitude was significant ($F(2,4) = 9.162, p < 0.05$) with bilateral lesion amplitude ($M = 2.94 \pm 0.74$) significantly lower than presurgery amplitude ($M = 10.79 \pm 0.90$). Slope exhibited a similar linear pattern ($F(2,4) = 12.295, p < 0.05$) with bilateral lesion slope ($M = 2.34 \pm 0.11$) significantly lower than presurgery slope ($M = 15.938 \pm 1.71, p < .05$).

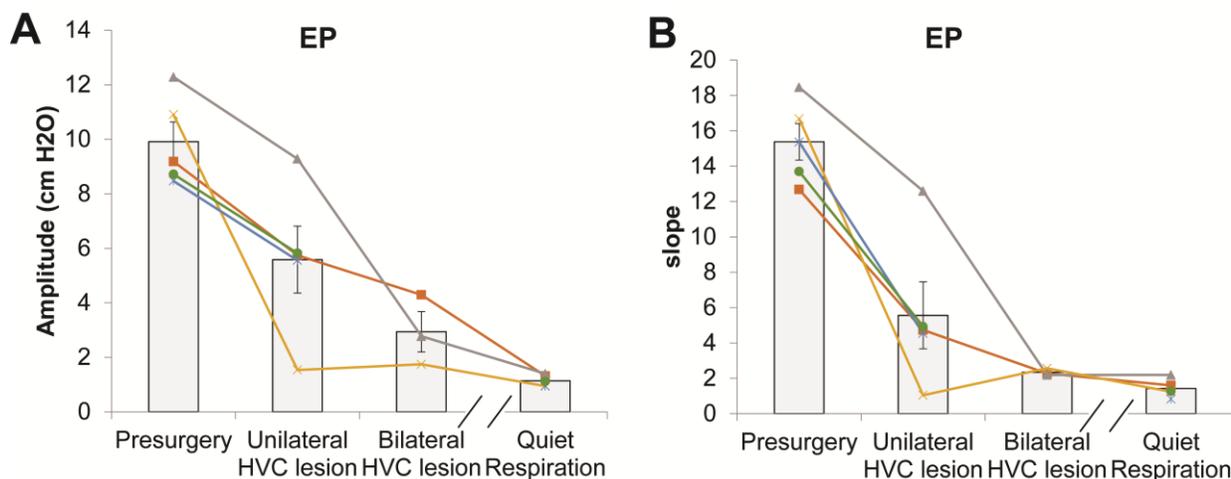


Figure 19. Linear decline in expiratory pressure features. Group mean \pm SEM for a) amplitude and b) slope. Individual data points illustrate all animals follow a similar pattern of decline after HVC damage. Average amplitude and slope of quiet respiration is provided to illustrate a lower boundary: respiratory activity presumably without active HVC participation.

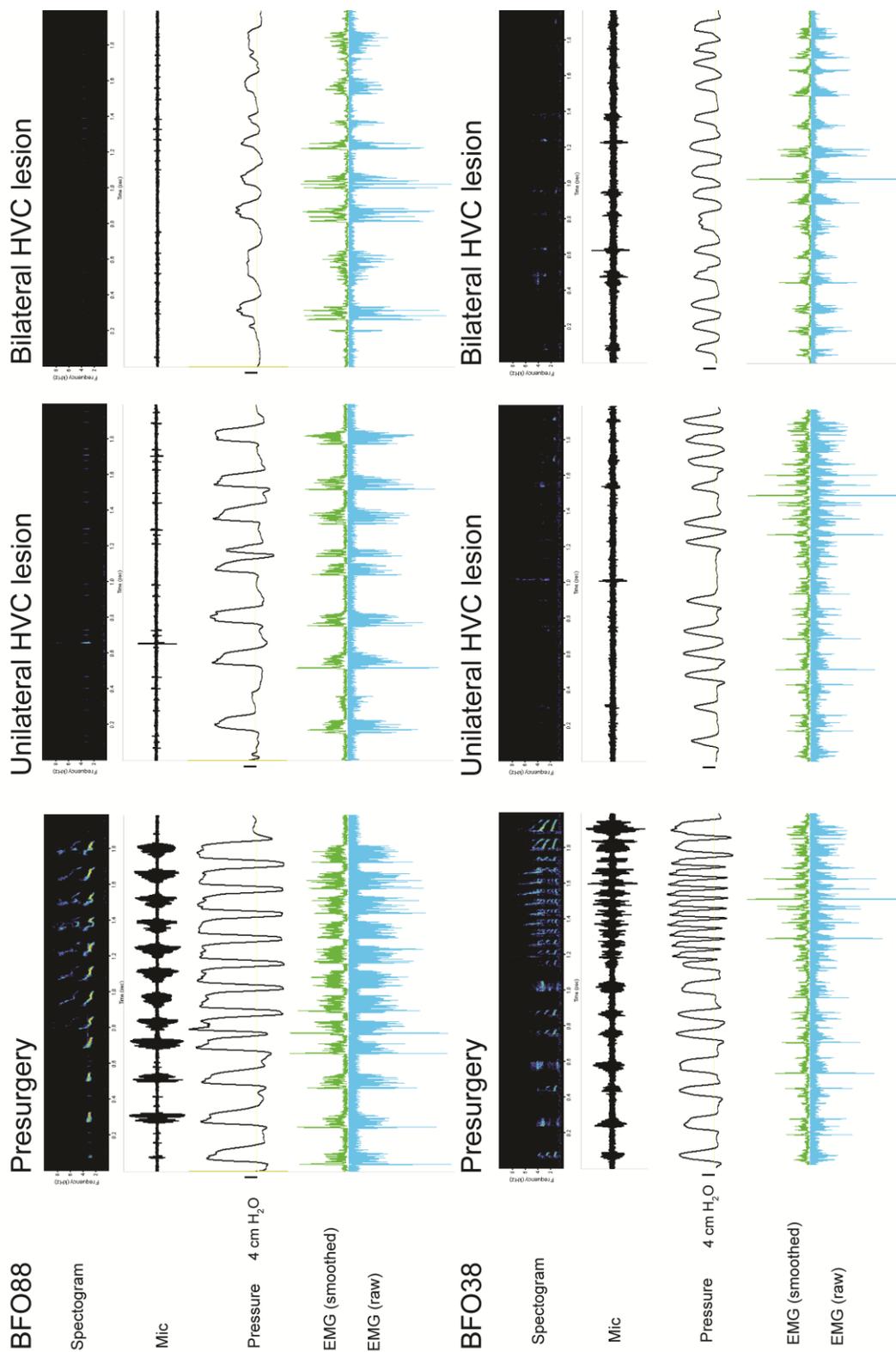


Figure 20. Stepwise reduction in expiratory pressure amplitude with sequential HVC lesion. Exemplar mic, pressure, and EXP EMG of two of the three sequential lesion animals. BFO88 was a unilateral right HVC lesion and BFO38 was a unilateral left HVC lesion. Acoustic amplitude decreased after HVC ablation and song elements are produced with a low sound

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amplitude. Amplitude of expiratory pressure pulses decreased after each HVC lesion. EMG data are not normalized in the figure, relative amplitude is displayed.

Linear decline in inspiratory pressure amplitude and slope. Unilateral HVC lesion reduced IP amplitude by $65\% \pm 0.07$ and IP slope by $63\% \pm 0.07$ (Figure 21). A repeated-measures ANOVA for IP amplitude showed a significant, linear decline ($F(2,4) = 14.437, p < 0.05$) with bilateral lesion amplitude ($M = -0.98 \pm 0.25$) significantly lower than presurgery amplitude ($M = -3.33 \pm 0.72$). Slope exhibited a similar pattern ($F(2, 4) = 9.973, p < 0.05$) with bilateral lesion slope ($M = -2.037 \pm 0.38$) significantly lower than presurgery slope ($M = -6.26 \pm 1.31$).

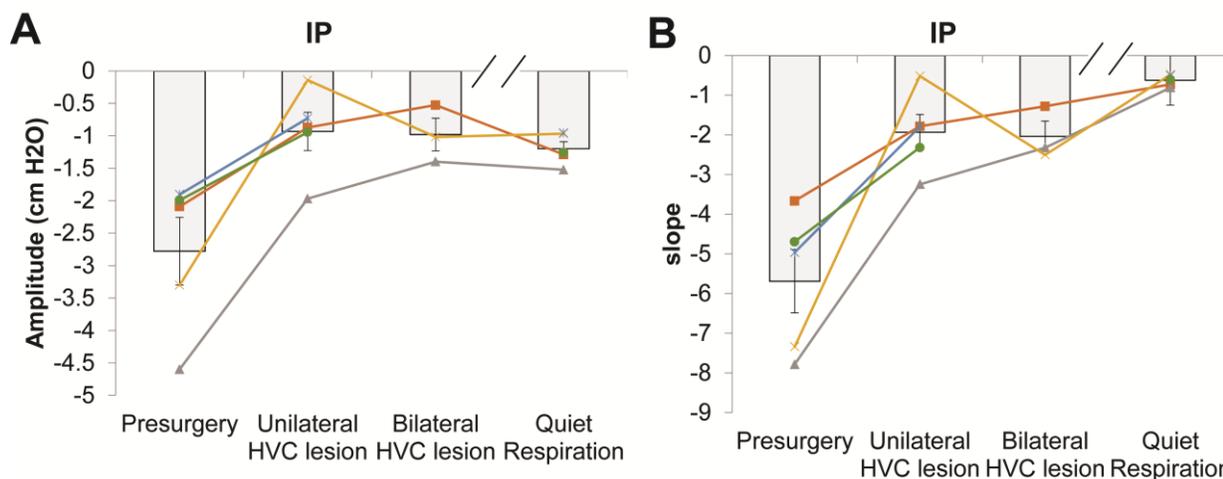


Figure 21. Linear decline in inspiratory pressure features. Group mean \pm SEM for a) amplitude and b) slope. Individual data points illustrate all animals follow a similar pattern of decline after HVC damage. Average amplitude and slope of quiet respiration is provided to illustrate a lower boundary: respiratory activity presumably without active HVC participation.

Unilateral HVC lesion reduces expiratory muscle (EXP) amplitude. In 4 out of 5 males, there was a decrease in EXP amplitude (Figure 22) after unilateral HVC lesion. I ran a t-test with all males and with BFO38 excluded (Table 5). In 4 animals, there was a significant decline in EXP amplitude.

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Bilateral lesions may produce an additional decline in EXP amplitude. However, BFO38 presurgery EXP amplitude was unusually low and we were unable to maintain EMG recordings from the third bilaterally lesioned male. With data across all three timepoints from only one male, we remain agnostic whether damage to both HVC results in further reduction of EXP amplitude (Figure 22A).

Table 5. t-test results for EXP features

	All subjects	BFO38 excluded
EXP amplitude (pre vs unilateral lesion)	$t(4) = 2.25, p = 0.08$	$t(4) = 13.91, p < 0.005$
EXP slope (pre vs unilateral lesion)	$t(4) = 1.28, p = 0.27$	$t(4) = 1.24, p = 0.30$

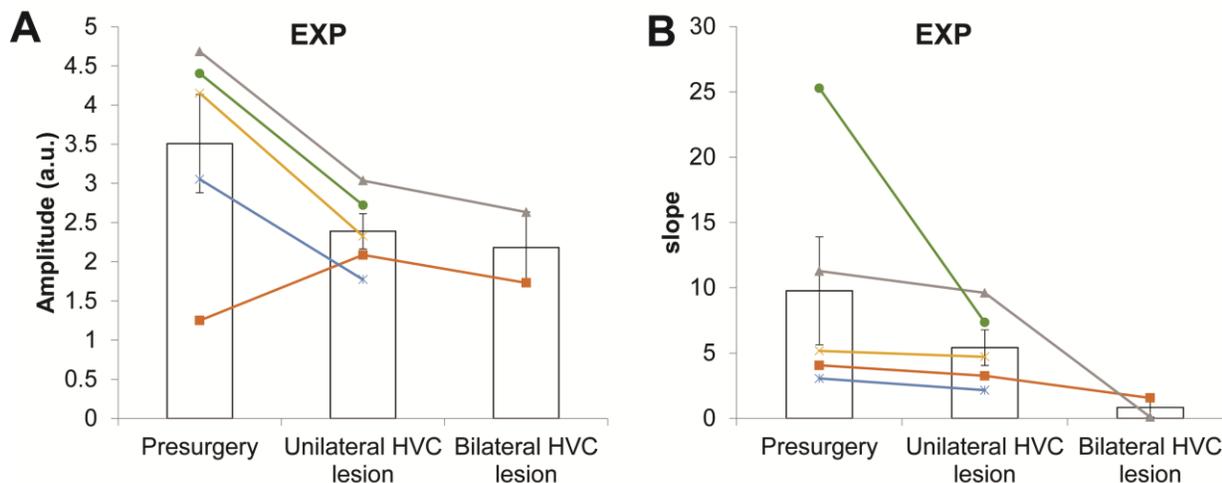


Figure 22. Unilateral HVC lesion reduces EXP amplitude. Group mean \pm SEM for a) amplitude and b) slope.

Unilateral HVC inactivation

The inherent limitation placed upon the sequential lesion technique is the delay that occurs after surgery as the animal recovers and regains the motivation to sing. We were concerned in two respects: EMG recordings fluctuate across days depending upon

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changes in the electrode's position in the body and potential decay of the signal. To an extent, we control for these issues by normalizing the amplitude (relative to quiet inspiration) and by using other behaviors (i.e. defecation, see Appendix F) to confirm our results are a function of HVC damage. However, to directly measure from syringeal and respiratory muscles in a repeatable manner, we chose to transiently reduce HVC output on a shorter timescale (hours) by implanting a microdialysis probe into HVC and "switch" HVC off and on by either infusing GABA-A agonist muscimol or saline. We collected air sac pressure data from nine males prior to, during and following inactivation. dTB and/or EXP electromyograms were recorded simultaneously during the inactivation protocol from a subset of these nine birds (dTB: $n = 6$; EXP: $n = 4$).

HVC inactivation transiently and systematically reduces EP amplitude and slope. Figure 23A-C illustrates the phase-specific reduction in EP amplitude. Pre-infusion and post-saline flush conditions are very similar in terms of acoustic and pressure features, whereas inactivation song shows altered acoustic features and lower amplitude EP pulses (relative to x-axis 10 cm H₂O). A repeated-measures ANOVA for EP amplitude was significant ($F(2, 16) = 22.55, p < 0.001$, Figure 23D) with the inactivation amplitude ($M = 7.34 \pm 0.82$) significantly lower than both presurgery ($M = 10.54 \pm 0.98, p < 0.001$) and flush ($M = 9.50 \pm 1.14, p < 0.001$) conditions. Presurgery and flush conditions were not significantly different from one another ($p = 0.11$). A repeated-measures ANOVA for EP slope was significant ($F(2, 16) = 23.88, p < 0.001$, Figure 23D) with the inactivation amplitude ($M = 7.88 \pm 6.62$) significantly lower than both presurgery ($M = 14.42 \pm 1.13, p < 0.001$) and flush ($M = 11.88 \pm 1.15, p < 0.001$) conditions. Flush conditions were marginally lower than pre-surgery song ($p = 0.06$).

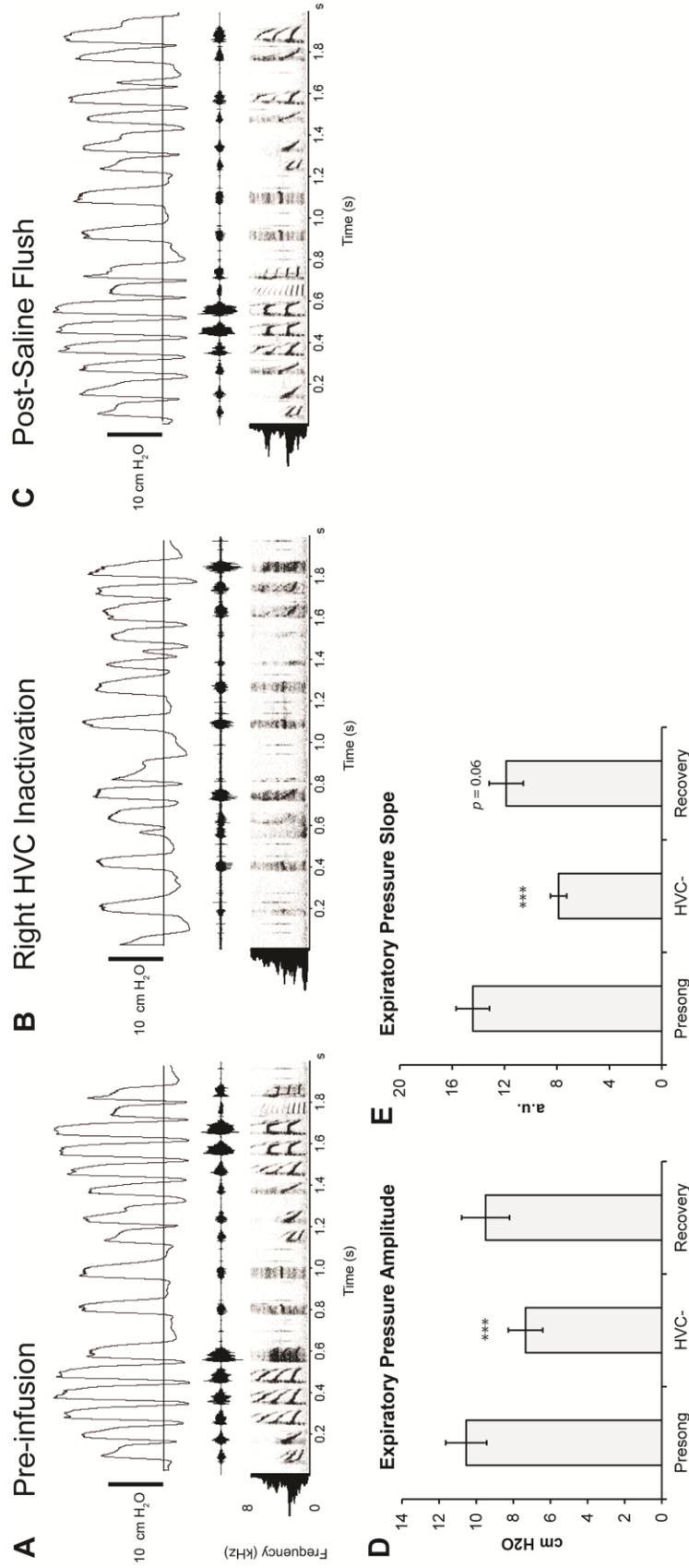


Figure 23. Transient decline in expiratory pressure features. Exemplar pressure and mic from a) pre-infusion, b) during right HVC inactivation, and c) recovery. B) HVC inactivation produces an altered pressure pattern and acoustic impairment that recovers after muscimol is flushed from the dialysis implant (C). D,E) Decline in EP amplitude and slope are largely restricted to the inactivation period (HVC-). $* p < 0.001$.**

Respiratory and syringeal muscle amplitude declined during HVC

inactivation. EXP amplitude replicated the effects of unilateral HVC lesion. During inactivation, there was a significant decline in EXP amplitude ($t(3) = 6.35, p < 0.01$) but not slope ($t(3) = 2.15, p = 0.12$, Figure 24A-B). Both dTB amplitude ($t(5) = 5.38, p < 0.005$) and slope ($t(5) = 2.30, p < 0.05$) were reduced by HVC inactivation (Figure 24C-D).

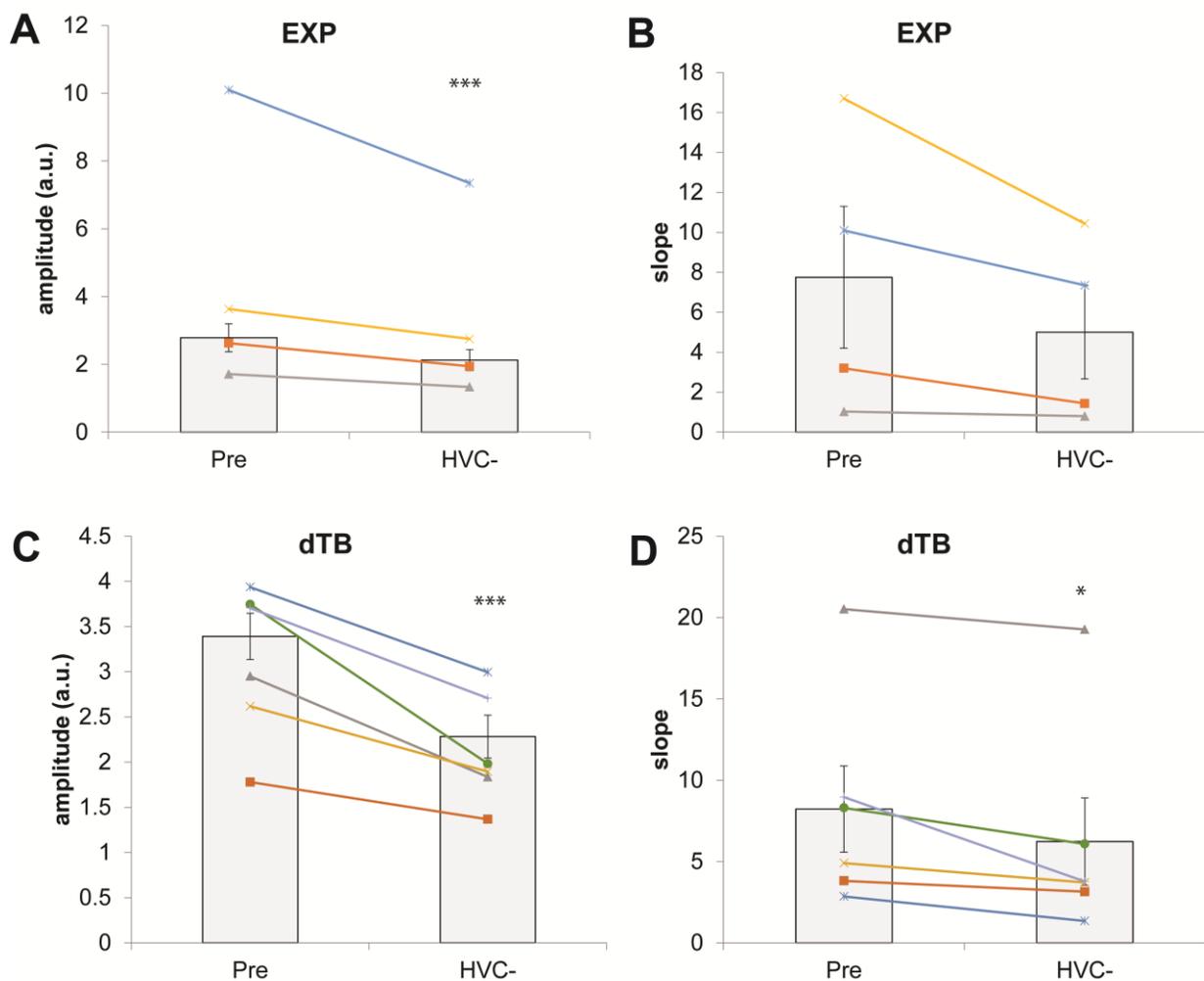


Figure 24. Unilateral HVC inactivation reduces EXP and dTB amplitude. Group mean \pm SEM of EXP a) amplitude and b) slope. C,D dTB amplitude (c) and slope (d) decline during inactivation. * $p < 0.05$, * $p < 0.001$.**

HVC inactivation alters the temporal relationship between dTB and EXP.

The third factor that influences EP pressure is whether there are temporal changes in when dTB and EXP activate. In a subset of three animals we were able to simultaneously record air sac pressure, dTB, and EXP EMGs during song. I measured the interval between the onset of dTB and EXP in 106 presurgery syllables and 100 inactivation syllables. Typically (with few exceptions), dTB activates first, and is measured as a negative value (Figure 25, black bars). During inactivation, this relationship changes drastically, with an increase in positive-value intervals (Figure 25, light blue bars, $p < 0.001$ K-S test). We observed a three -fold increase in the coefficient of variance (Pre: 0.87, Inactivation: 2.94).

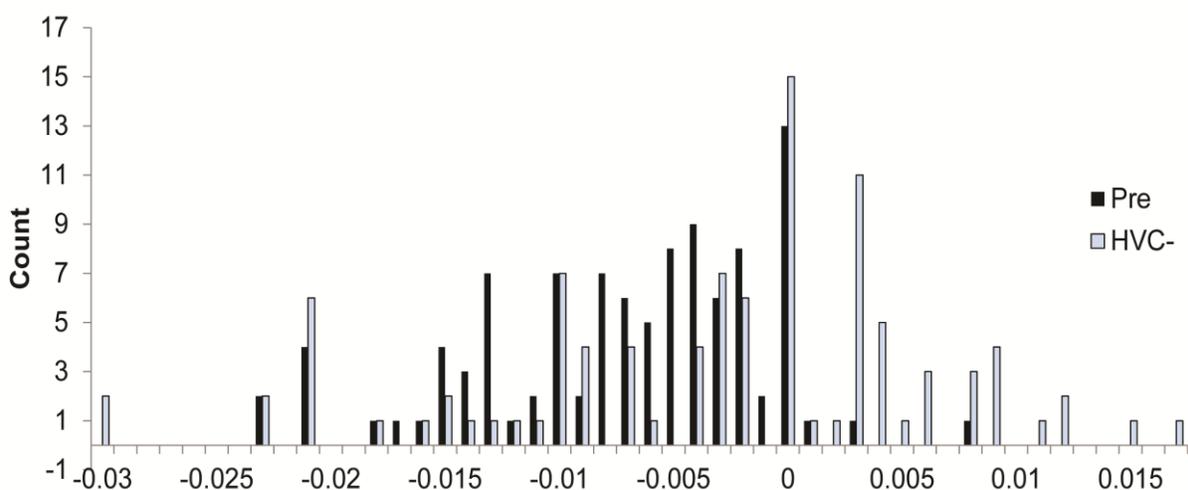


Figure 25. HVC inactivation can result in positive dTB to EXP onset intervals. Histogram of dTB to EXP onset interval of pre-inactivation (black) and inactivation (blue) syllables. Typically, dTB fires first, which results in a negative dTB to EXP onset interval. However, inactivation increases the probability of dTB occurring after EXP onset.

Muscle amplitude is positively correlated with pressure features. In the same subset of syllables, we found that both dTB and EXP amplitude had a strong positive relationship with EP amplitude and slope (Figure 26A-B,E-F). We were

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interested in whether similarity in the EMG profile was important. We measured the cross-correlation between dTB and EXP waveforms and found a moderate relationship with EP amplitude and slope (Figure 26C,G). We did not find evidence that onset interval was directly related to EP amplitude and slope (Figure 26,D,H).

During inactivation, dTB and EXP amplitude and slope retained a strong relationship with EP amplitude (Figure 27A-B,D-E). EMG cross-correlation was no longer related to pressure features (Figure 27C,F).

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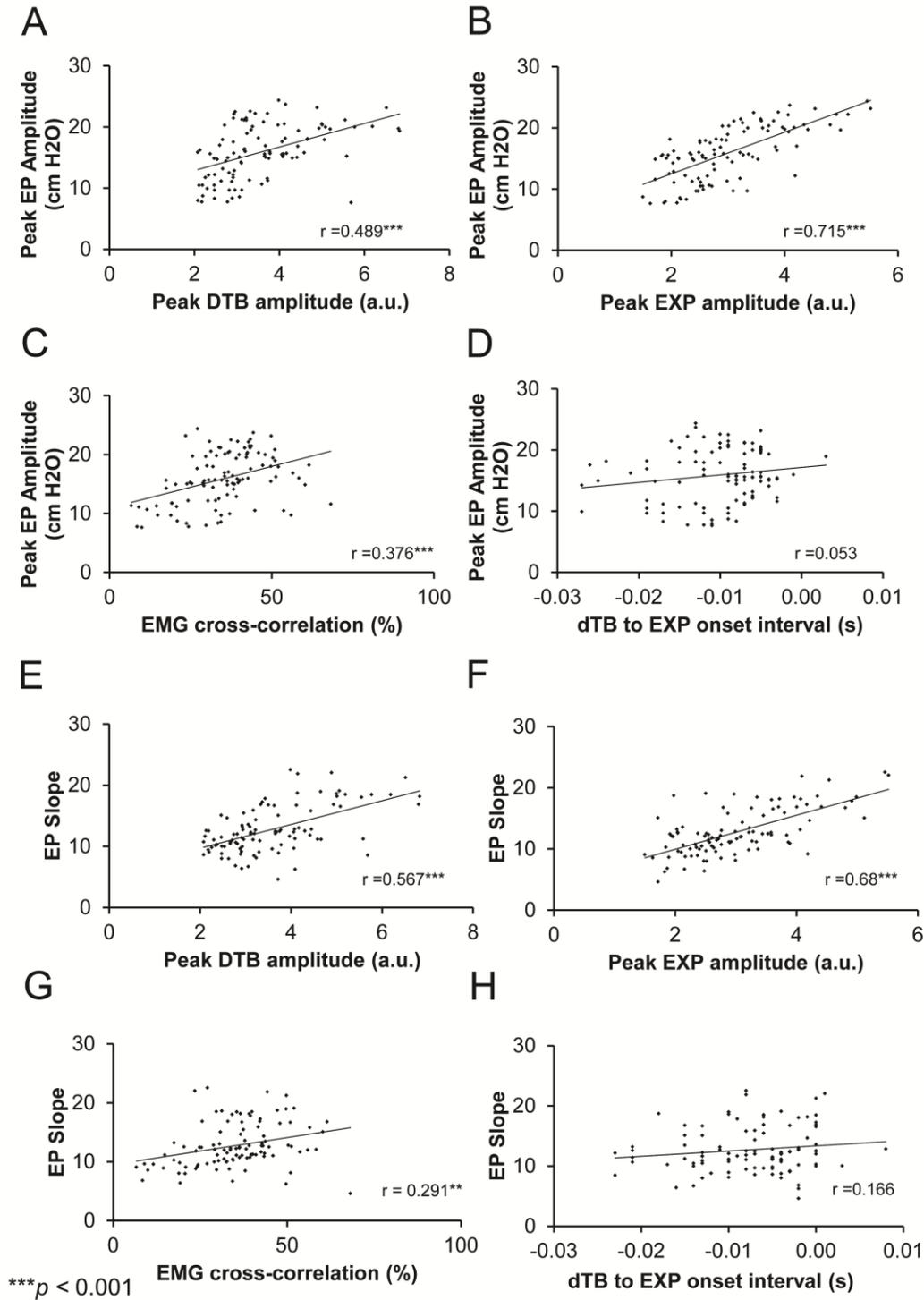
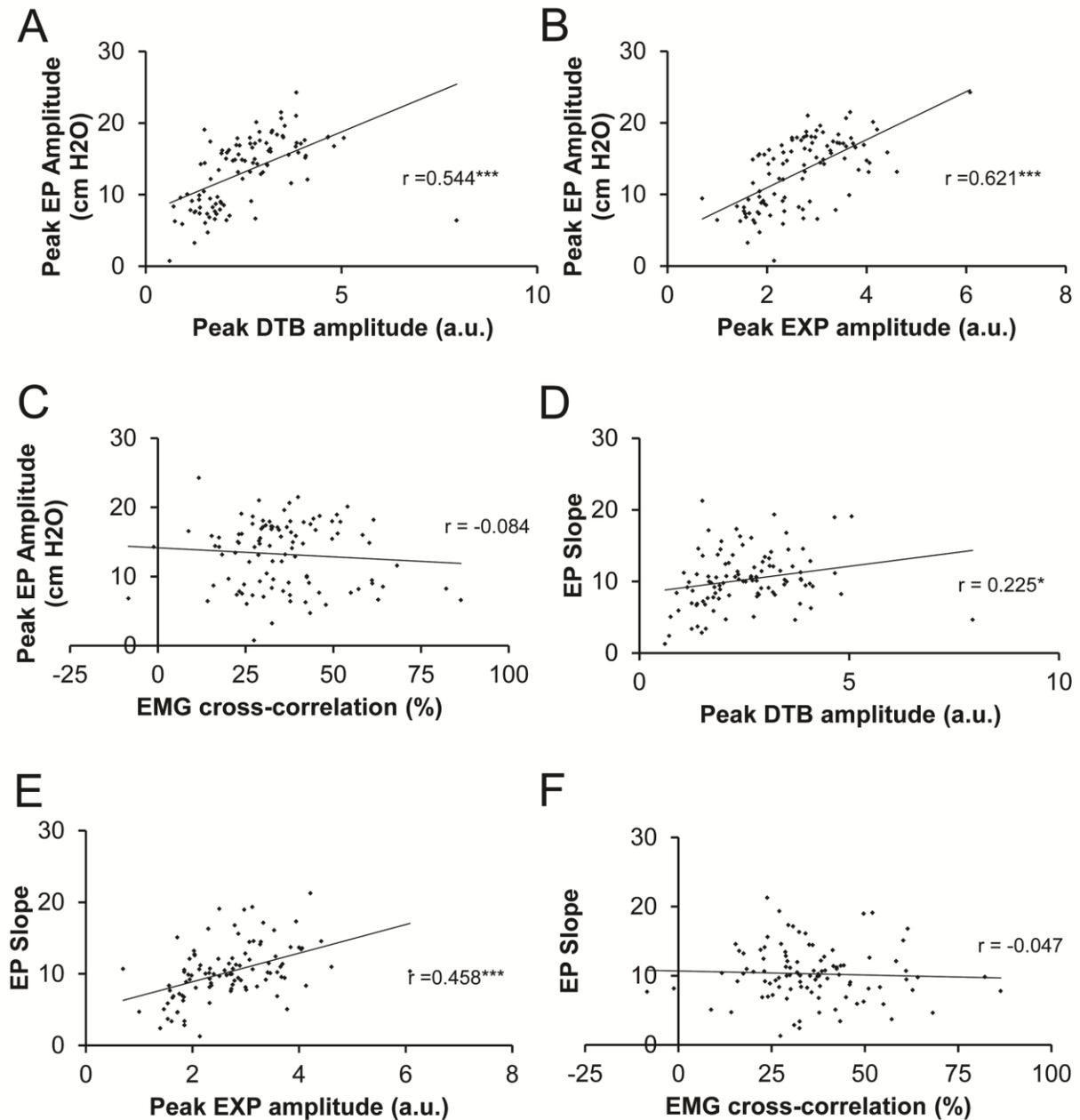


Figure 26. Correlation between pressure and muscle activity in pre-inactivation syllables. A,C) EP amplitude is significantly correlated with muscle EMG amplitude and dTB to EXP cross-correlation. D) onset interval is not significantly correlated with EP amplitude. E-G) EP slope is significantly correlated with muscle EMG amplitude and dTB to EXP cross-correlation. F) onset interval is not significantly correlated with EP slope. ** $p < 0.01$. * $p < 0.01$**

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*** $p < 0.001$

Figure 27. Correlation between pressure and muscle activity in HVC-inactivated syllables. A,B) EP amplitude is significantly correlated with muscle EMG amplitude and dTB. C) EMG cross-correlation is no longer correlated with EP amplitude. D-E) EP slope is significantly correlated with muscle EMG amplitude and dTB. F) EMG cross-correlation is no longer correlated with EP slope. * $p < 0.05$, *** $p < 0.001$

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Summary and Discussion

In oscines, phonation is directly affected by syringeal muscle activity and respiratory muscle activity. We have established that HVC output to downstream forebrain and brainstem nuclei is directly related to the underlying song respiratory motor pattern. Using a combination of electrolytic lesion, drug infusion, and physiological recording techniques, we were able to uncover changes to muscle activation and the temporal relationship between two muscles that are actively engaged during sound production. Thus, we demonstrate that HVC manipulations affects both respiratory and syringeal motor systems, which are critical for phonation.

Left and right HVC nucleus independently contribute to respiratory features

During quiet respiration, approximately 1-1.5 cm H₂O of pressure is consistently generated. Song respiration is easily recognizable by a minimum two- to three-fold increase in pressure. We tested the sequential lesion effects on EP amplitude and slope against a linear model and found both to be significant ($p < 0.05$). This would mean each HVC nucleus generates central input that drives an equal proportion of the EP pulse. Further, with both HVC lesioned, EP amplitude and slope remain higher than quiet respiration and song is not entirely abolished. These results are in direct contrast to initial HVC lesion work that concluded bilateral HVC ablation eliminated song (Nottebohm, 1976). It also demonstrates that HVC is not entirely responsible for respiratory motor control. It most likely shares the work with RA and/or downstream midbrain nuclei.

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Song rhythm generator or system synchronizer?

An issue that is hotly debated among songbird neuroscientists is the nature of HVC's contribution to song production and song structure. A prominent voice in the field has argued that HVC is a reliable, but independent, tempo machine that maintains song stereotypy by firing at reliable intervals and setting populations of RA neurons into motion (Long, et al., 2010). A contrasting view is that HVC's firing pattern is related less to tempo and more involved in ensuring precise coordination between respiratory, syringeal and upper vocal tract systems (Amador, et al., 2013).

Resolving this issue is outside the scope of this study as they involve paired recording from HVC and RA in singing birds. Our results do provide insight into key declarations. Although we do not differentiate between HVC-RA and HVC-Area X (song pathway critical for song learning) output, the Area X-basal ganglia-LMAN pathway is less likely to be involved in motor production.

Fee and colleagues have asserted that HVC-RA activity is not directly related to acoustic features, rather to controlling song timing. Our results suggest otherwise. We demonstrate that direct manipulation of HVC output to downstream brain regions produce a repeated, systematic decline in muscle activity, and this decline is strongly correlated with the amplitude of air sac pressure. Although they have analyzed changes in pressure features during song learning and maturation, they have concluded that bilateral HVC lesions do not strongly affect syllable expirations and inspirations (Veit et al., 2011). Our results can co-exist with his published findings as he acknowledges there was a reduction in amplitude and his study did not measure the slope of EPs and IPs.

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Fee and colleagues have also repeatedly demonstrated that local HVC networks operate in a highly reliable manner and regulate temporal features of song. Our results are in complete agreement with this. HVC inactivation using muscimol would increase interneuron activity, thus disrupting any tightly regulated local networks. As a result of this disruption, we altered the temporal relationship between dTB and EXP muscle activation. The results are consistent with the view that this occurs due to a delayed activation of dTB motor units affecting the initial activation onset, slope and the muscle activation amplitude (Figure 24C-D).

Amador and colleagues have asserted that HVC-RA activity serves to synchronize relevant motor systems. Our results are in complete agreement. Once again, the inactivation-specific decoupling of dTB and EXP onset strongly suggest that HVC plays an important role in keeping the syringeal and respiratory systems synchronized for efficient gating of airflow. HVC-RA projection neurons are diffusely organized in RA itself, even though RA is subdivided into a syringeally-controlling dorsal portion and a respiratory-controlling ventral subdivision (Vicario, 1991). At any given time, HVC-RA neurons may be driving discrete ventral and dorsal populations of RA neurons simultaneously.

Amador and colleagues also assert that interneuron activity, which is normally tonically active, is uniquely suppressed within 10 ms of a vocal motor gesture. I find this interesting as muscimol would produce the exact opposite effect on interneurons. Taken together, I would hypothesize that interneurons act as the gatekeepers of excitatory HVC activity. One projection neuron could project onto both ventral and dorsal RA neurons or sets of HVC-RA neurons could be firing in tandem. In the first scenario,

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variations in interneuron activity could be regulating local networks, to allow sparse HVC-RA firing. In the second scenario, interneurons could be coordinating local networks so that multiple HVC-RA neurons fire to dorsal and ventral RA targets independently, but simultaneously.

It was recently proposed that a method for resolving the feed-forward and GTE models was by determining whether HVC-RA neuron activity is time-locked to active singing (Picardo, et al., 2016). Picardo and colleagues proposed that because HVC-RA neurons fire equally during silent intervals and during phonation, there was evidence to suggest HVC-RA activity was unrelated to coordinating respiratory, syringeal and oro-esophageal systems. However, dTB and EXP activation must always occur prior to phonation and our findings would explain why Picardo et al. found equal bursting probability over the course of singing a bout or motif. Further, our results suggest that an intermediate model is viable – that HVC activity represents both movement and elapsed time (Picardo, et al., 2016).

Chapter 5: General Discussion

In the early 2000s, a summit of geneticists, microbiologist, anatomists and physiologists collaborated to produce a definitive manuscript on the anatomy and structure of the avian brain. A central limitation to generalizing findings in the avian community has been the argument that the avian brain is fundamentally different from the mammalian, and ultimately human, brain. Thus, the goal and the eventual value of this publication was to revamp nomenclature of avian brain structures based on electrophysiological activity, neuron type, key neurotransmitters, and more, to make songbird neuroscience more accessible to other scientists.

Prior to Reiner et al., (2004), the acronym HVC was synonymous with its former anatomical name *hyperstriatum ventral pars caudalis*. Another term used was Higher Vocal Center, a term I can trace back to Fernando Nottebohm's 1976 publication that lay the foundation for the song system nuclei. It has since come to light that HVC does not occupy the hyperstriatum as Nottebohm thought, but is actually part of the pallium. As research has come to understand the circular and recurrent nature of oscine song circuitry, the view that HVC is the "top" of the hierarchy is not necessarily true, thus stating that HVC is the "Higher Vocal Center" is not accurate (Margoliash, et al, 1994; Schmidt, 2004). Nevertheless, its importance for song maturation and adult song production has been demonstrated repeatedly and I would argue that for many songbird neuroscientists and behaviorists, "HVC" is so well-recognized as a key nucleus or linchpin brain region for communication that it has proven difficult to think of it as anything else. Regardless, Reiner, et al., (2004) ultimately suggested that it would keep

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the letters, but it would be a proper name, unassociated with the incorrect anatomical name or Nottebohm's coined phrase.

Overall, the findings presented in this thesis fall in line with the traditional view of HVC as a critical region for song production. In this chapter, I will discuss HVC's role in phonation, implications for song learning and recovery, and the interplay between neural and peripheral lateralization.

Forebrain nuclei to muscle to air pressure to sound

Across several experiments, we provide direct evidence for how forebrain signals are translated into sound. In experiment 1, we presented data illustrating the relationship between unilateral HVC lesion and resultant reduced song amplitude. Experiment 2 illustrated that amplitude will gradually recover months after large HVC lesion. Experiment 3 shed light on why songs are sung more quietly: the underlying reduction in EP amplitude and slope that, interestingly, does not recover months after surgery. Experiments 4 and 5 looked at potential peripheral mechanisms for the altered respiratory pattern and demonstrated that reducing HVC input (via lesion or inactivation) does two things: directly attenuates muscle activation and decouples the onset of dTB and EXP muscles. Altogether, these studies paint the following picture:

Brainstem and midbrain nuclei regulate quiet expiration and inspiration. In singing birds, HVC, RA and downstream nuclei contribute to engage syringeal and respiratory motor units. It is very likely that the number of neurons/ coactive neuron populations involved is directly related to the number of muscle motor units that are engaged. I propose this is the case as unilateral and bilateral HVC lesions result in a stepwise decrease in EP amplitude and slope (Figure 19, 20).

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We confirm that contraction of expiratory muscles is a necessary step for proper vocal production. The magnitude of HVC output to downstream nuclei has a direct influence on the amplitude of expiratory muscle activation (Figure 22A, 24A). Reduced activation would generate lower pressure head that may be insufficient to force vocal tissue to vibrate, especially as muscle activation in the adductor, dTB, is also reduced. Interestingly, this is in direct contrast to a related manipulation: unilateral denervation of syringeal muscles by resection of NXIIIts – the cranial nerve that directly innervates the syrinx - does not produce a similar effect (Figure 28, Urbano, et al., 2013). It is possible that males are able to maintain stereotypical air sac pressure patterns with the contralateral, normally functioning adductor muscle so long as expiratory muscle activation is intact. This is an explanation that supports data presented in this document and highlights the importance of normal expiratory muscle function.

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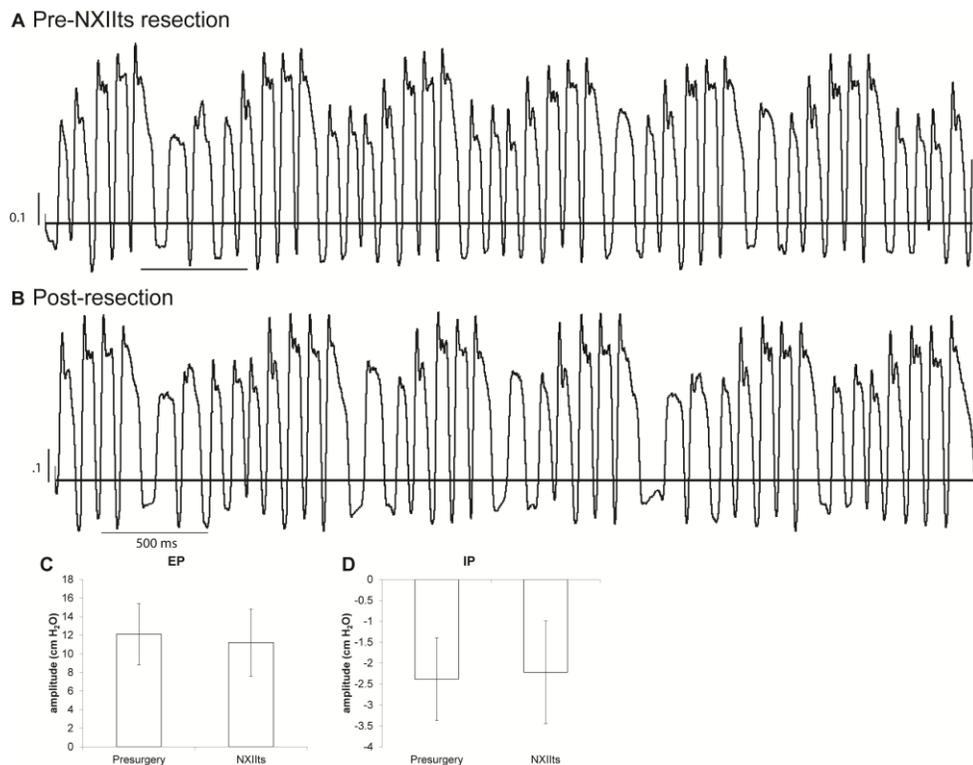


Figure 28. Unilateral tracheosyringeal nerve resection does not alter the respiratory motor pattern. A) Air sac pressure pattern of a pre-surgery song. B) Air sac pressure pattern after nerve resection looks very similar to pre-surgery patterns and absence of extended silent periods and low amplitude pressure pulses. C,D) Pre and post resection amplitude is unaffected for c) expiratory and d) inspiratory pulses.

Equally important for phonation is normally functioning adductor muscle, which is responsible for moving vocal tissue into the bronchial lumen to “gate” or constrict the airway into the upper vocal tract. As mentioned in Chapter 4, a pressure differential above and below the vocal tissue creates a Bernoulli effect that pulls air through the opening and the constant exchange in positive and negative pressure is what causes vocal tissue to vibrate against each other and transform the mechanical energy into sound. We have presented that reduction in either amplitude or slope can impair gating (Figure 24C-D). Thus, quiet song and the underlying altered respiratory pattern can be attributed to changes in syringeal and respiratory muscle activity.

The role of HVC in achieving adult-type respiratory motor pattern

In chapter 3, we briefly discussed the ontogeny of expiratory and inspiratory pulses from subsong to adult song and that one function of song rehearsal in juvenile songbirds is to improve and achieve stereotypy of syringeal and respiratory motor systems. The key differences between subsong and adult song are 1) both acoustic and pressure characteristics of subsong syllables are highly variable, 2) subsong syllables are lower sound amplitude and produced at lower EP amplitude, and 3) lack a tight 1-1 relationship between audible sound and expirations (Veit et al., 2011; Cooper, et al., unpublished observations, Figure 29). To further explain point 3, I refer to the air sac pressure pulse and matching song spectrogram in figure 29A. Note how each supra-ambient EP pulse is filled with a combination of audible vocalization and silence. As juveniles rehearse and tighten this relationship between sound and expiration, a greater portion of the EP is filled with sound (Veit et al., 2011; Figure 29B). At the same time, the acoustic structure is undergoing further refinement and will be produced with greater bout-to-bout stereotypy. As adults, EPs will be entirely filled with sound and onsets and offsets of syllables and their underlying expiration will be tightly correlated.

Veit et al., 2011 performed bilateral HVC lesions on mid- plastic zebra finch juveniles (46-66 dph) and reported loss of HVC reverted to subsong: soft, highly variable vocalizations with lower EP amplitude and longer duration EP pulses. We observed a similar pattern of results after unilateral HVC lesion or inactivation. In a subset of three animals wherein we successfully lesioned both HVC, song was nearly inaudible (Figure 20). Scatterplots of individual song syllables are extremely similar to subsong syllable distribution in that tight clusters of individual syllables are not retained

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(data not shown). It is not difficult to imagine that early studies concluded bilateral HVC lesions mute song; birds sing so softly that the data cannot be reliably recorded using an amplitude threshold trigger.

Our results suggest that HVC maintains adult-“typical” levels of sound amplitude by driving respiratory and expiratory muscles and doing so in a highly synchronized manner. These findings suggest that in order to do so as adults, it is critical for HVC to encode and refine relevant motor commands as juveniles through repeated trial and error. Ablation of neurons that encode specific syllables/motor gestures or the disruption of local HVC networks via hyperexcitation of interneurons reverts this development, thus bringing out “subsong”-like characteristics.

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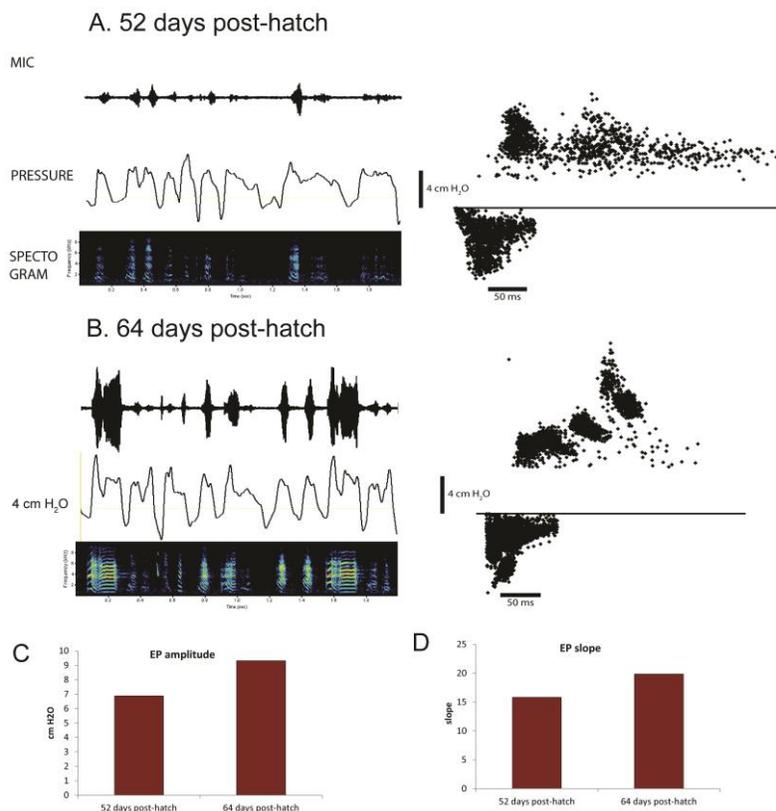


Figure 29. Juvenile song rehearsal increases stereotypy of expiratory pressure pulses. A) Exemplar mic and pressure of a juvenile song motif at 52 dph (*left*). Scatterplot of individual song syllables (*x-axis*: time, *y-axis*: amplitude) do not show clusters based on syllable identity, a feature of adult song syllables. **B)** Exemplar mic and pressure of a juvenile song motif at 64 dph (*left*). Scatterplot of individual song syllables show greater differentiation based on syllable identity. There are now 3-4 syllable clusters. **C,D)** EP amplitude and slope at 64 dph is higher than EP amplitude and slope at 52 dph.

HVC and vocal recovery

Following the recovery of acoustic and pressure characteristics 5+ months after HVC lesion demonstrates that although syllable duration and amplitude recover, ISI never does. Pressure features never recover at 6-8 months post lesion and it is likely dTB and EXP activity were permanently affected.

Within-day similarity scores at PSM 5 are similar to within-day presurgery scores (pre: 60-80%; PSM5: 70-80%), indicating syllables are produced in a stereotyped

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manner. While we do not know whether EXP and dTB ever recover following HVC lesion, given the acoustic stereotypy, compensation must occur at either the syrinx or upper vocal tract. Regardless, the dissociation between vocal motor and respiratory motor recovery is very interesting as it implies a potential mechanism for vocal compensation that may be relevant for individuals with permanent respiratory deficit.

Neural and peripheral lateralization

We have previously demonstrated that syringeal vocal tissue and supporting structures have anatomical and microstructural asymmetries. The left vocal tissue are specialized for producing higher frequencies and the right vocal tissue is better suited for producing chaotic, lower frequencies (Urbano, 2013). The initial drive behind this research project was to determine whether the Bengalese finch exhibited evidence of neural lateralization or if its lateralized vocal production was solely due to anatomical and microstructural asymmetries. By directly comparing the effects of left and right HVC lesion on vocal recovery, syllable types produced, and acoustic and temporal features, we were able to find patterns in the data that point towards features that rely on the intact left HVC.

Experiments 1 and 2 established that left HVC damage produces the greatest decline in peak frequency and amplitude (Figure 9, 10B). Given that in the Bengalese finch, peak frequency and amplitude are highly correlated, it can be argued that the decline in peak frequency is due to impaired respiratory motor pattern (Secora et al., 2013). Indeed, right HVC damage also produces a decline in peak frequency and amplitude, although not to the same degree, and experiments 3-5 established a direct relationship between HVC and song-related respiration. Thus, it is possible that an

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inability to regulate or induce vibrations in the left vocal tissue is responsible for the drop in peak frequency.

However, the trajectory of recovery is different based on side of lesion. The rate of vocal recovery over the first week showed right HVC lesions recover more quickly (Figure 11B). At PSM 1, right HVC lesion animals recover amplitude (Figure 12C) and by PSM5, largely recover peak frequency (Figure 13B). Left lesion animals are slower to recover amplitude, but do so by PSM 5. Peak frequency, however, remained attenuated. As EP amplitude does not differ between left and right lesions at PSM6-8 (Appendix E), the delayed acoustic recovery after left lesion cannot be attributed to impaired respiratory motor control.

An interesting pattern that emerged from observing song changes over a five month period is that songs at PSM 5 achieve motor recovery, but are inherently different from presurgery song (see appendix B). Higher frequency syllables are missing in three out of four left lesion males. With the exception of one abnormally long low frequency stack (BFW50 PSM 5, appendix B), syllable duration has recovered (Figure 13E). Syllable amplitude has similarly returned to baseline levels. In an animal species that produces one, largely stereotyped song all its life (Cooper et al., 2012), this pattern suggests that song is permanently changed by HVC lesion.

Taken together, these results support a model of left HVC dominance for motor recovery and critical for higher frequency syllables. Which begs the question of what is the function and relevance of specialization in only left HVC when vocal tissue lateralization gives the left and right sides unique frequency characteristics?

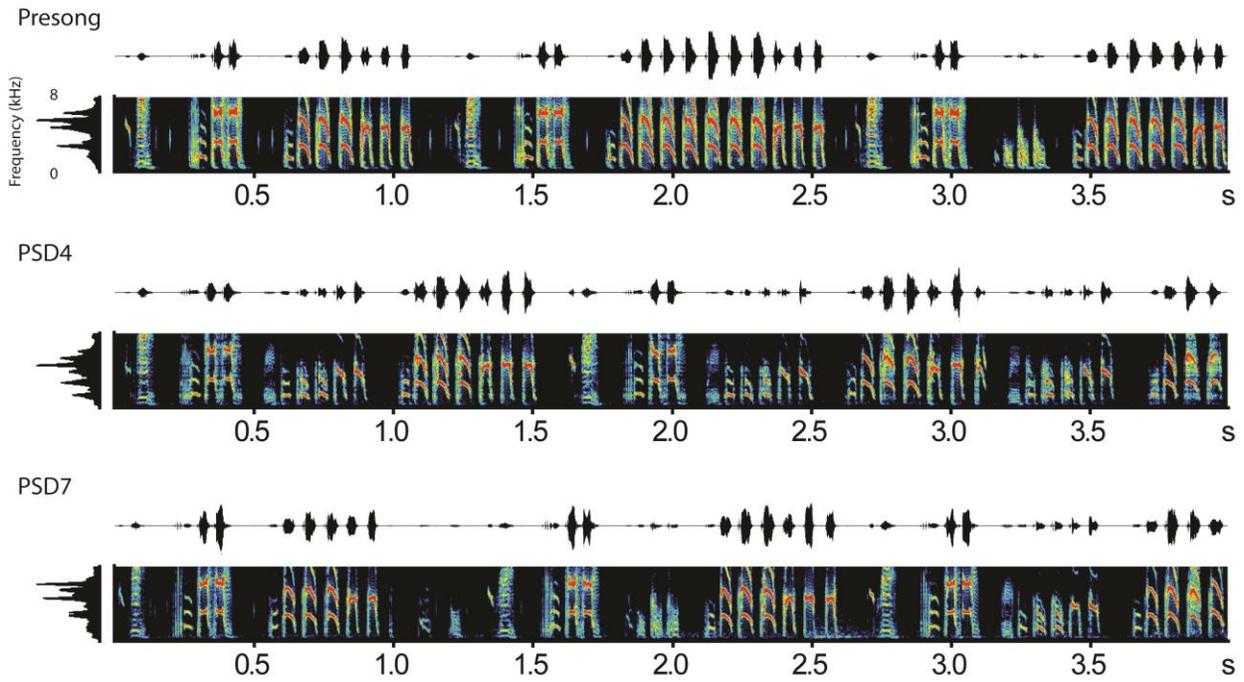
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One possible explanation is related to the mass of the left and right vocal tissue. The right lateral labium (LL_R) is large and easily occludes the bronchial lumen; minimal neural input may be required to set up the respiratory-syringeal system into the right configuration. In contrast, left lateral labium is one-third the size of LL_R and manipulating it to not only occlude the bronchial lumen, but to also vibrate at higher rates and maintain control under greater tension may require complex neural input (Riede & Goller, 2014). Mutation of HVC to efficiently control the left sound source would potentially enable the Bengalese finch to widen the acoustic complexity of their song while minimizing variance in neural control.

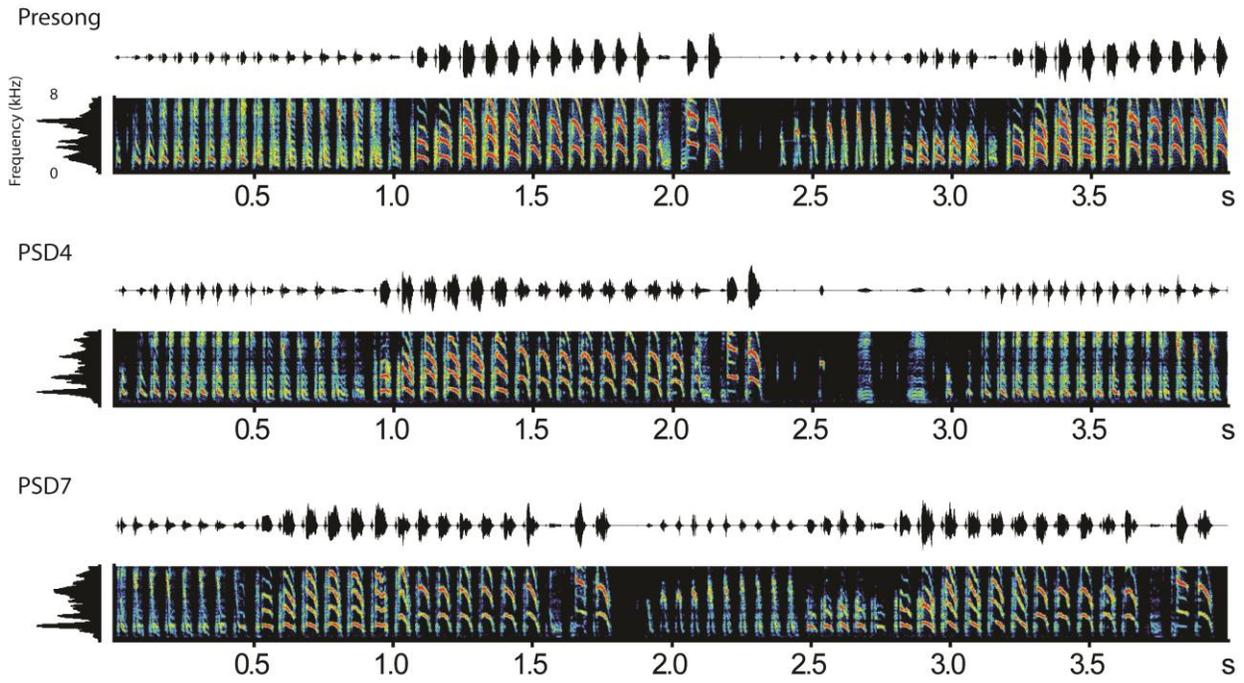
In conclusion, we have presented evidence of lateralized motor control in HVC of the Bengalese finch. We have also provided novel evidence that HVC regulates temporal features via activation of syringeal and respiratory muscles. These results illustrate the importance of a forebrain area that overrides respiratory and vocal motor nuclei in the midbrain, a phenomenon that is currently unstudied in the mammalian brain. Future research can extend these findings by 1) investigating the dichotomy of respiratory and vocal motor recovery or 2) investigating mammalian forebrain areas involved in synchronizing laryngeal and respiratory motor systems in order to produce innate vocalizations and mechanisms for overriding autonomic physiological processes.

Appendix A: HVC microlesion song spectrograms

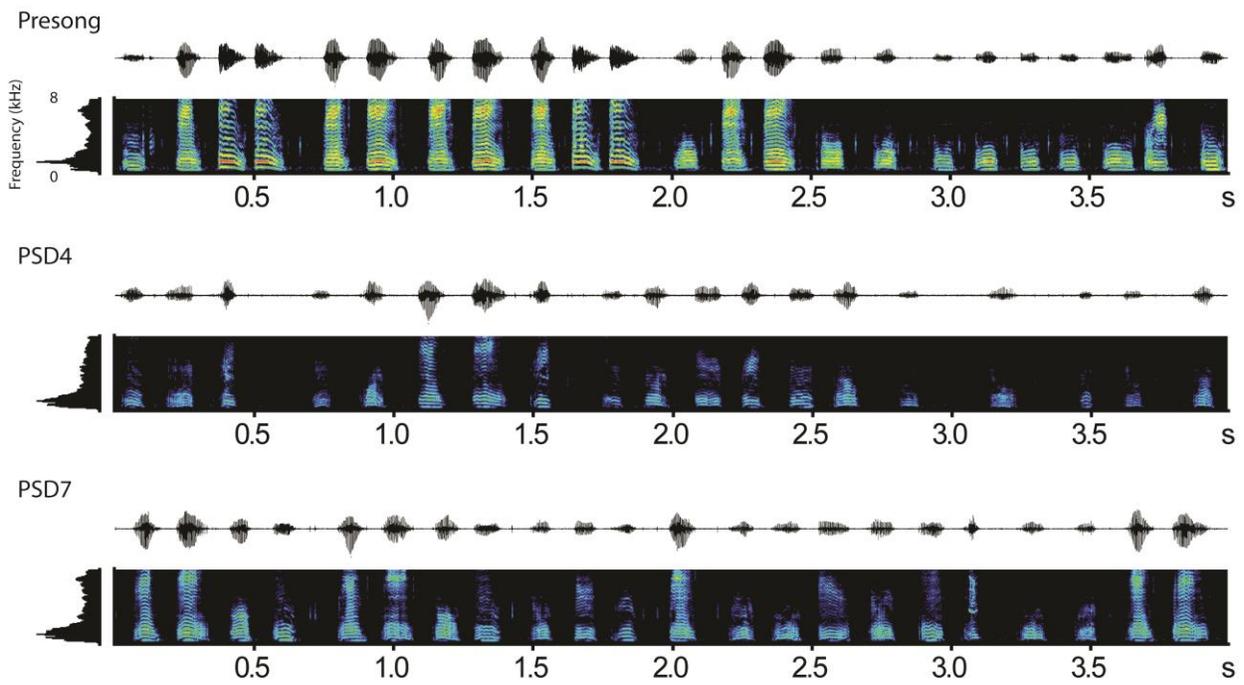
BFW55 Left HVC microlesion



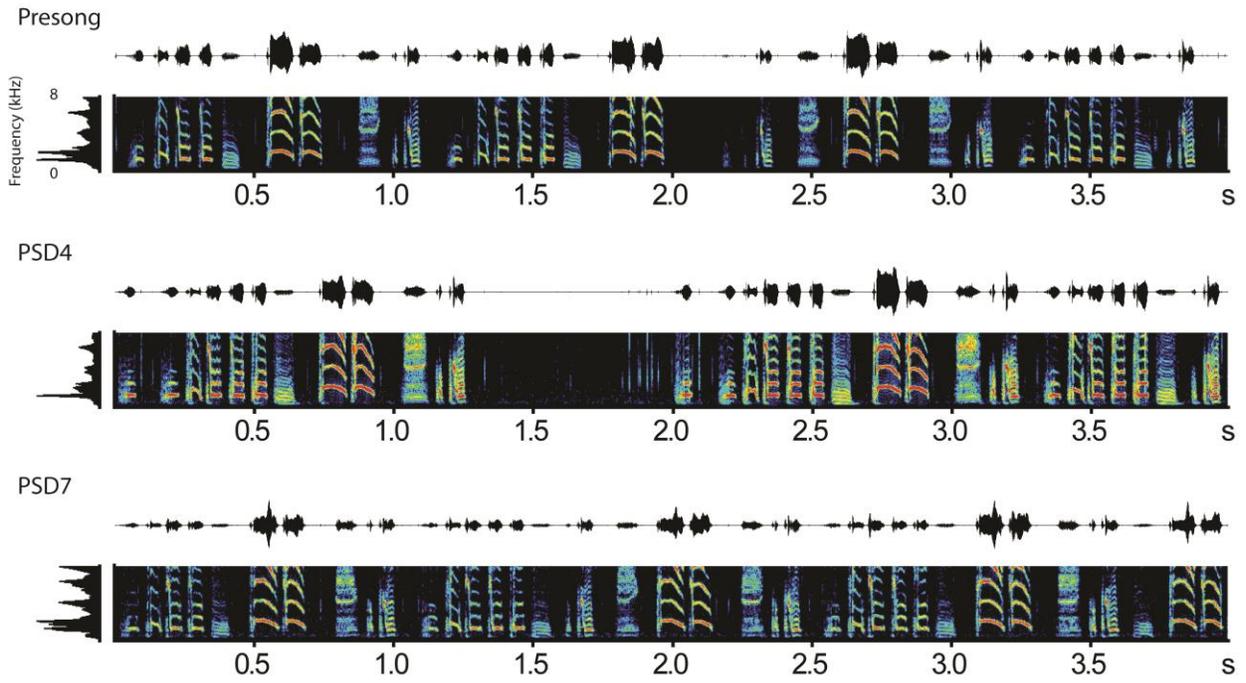
BFW64 Left HVC microlesion



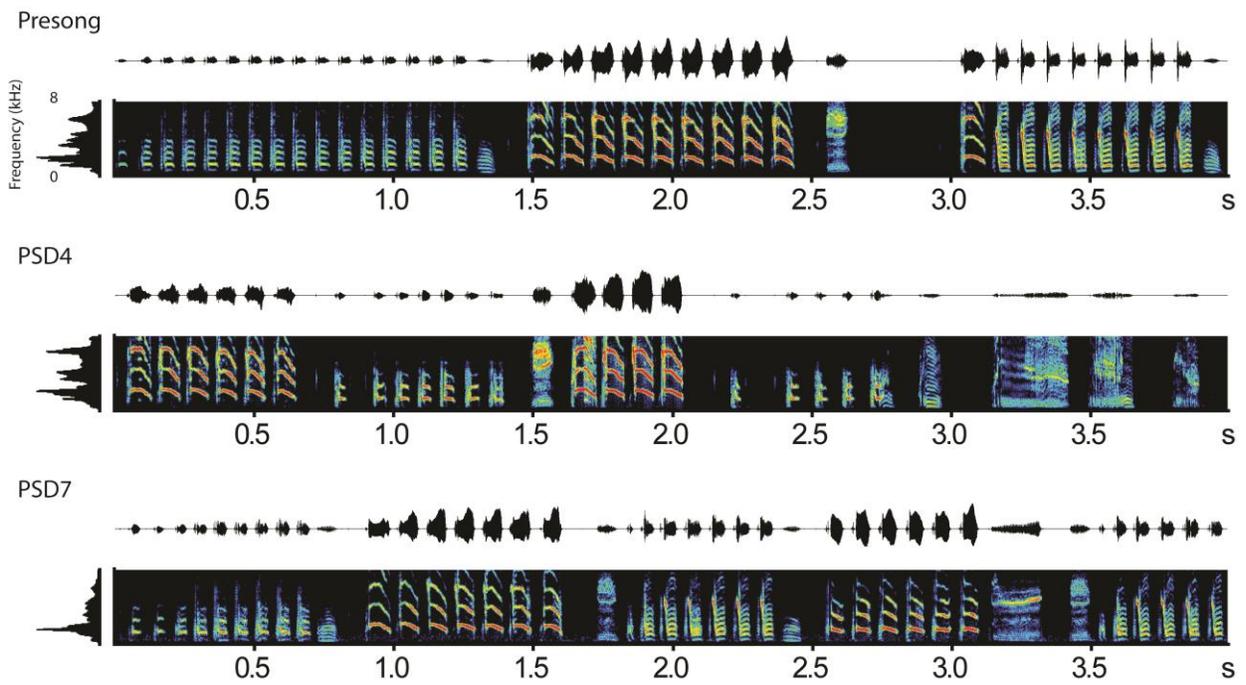
BFP31 Right HVC microlesion



BFW33 Right HVC microlesion

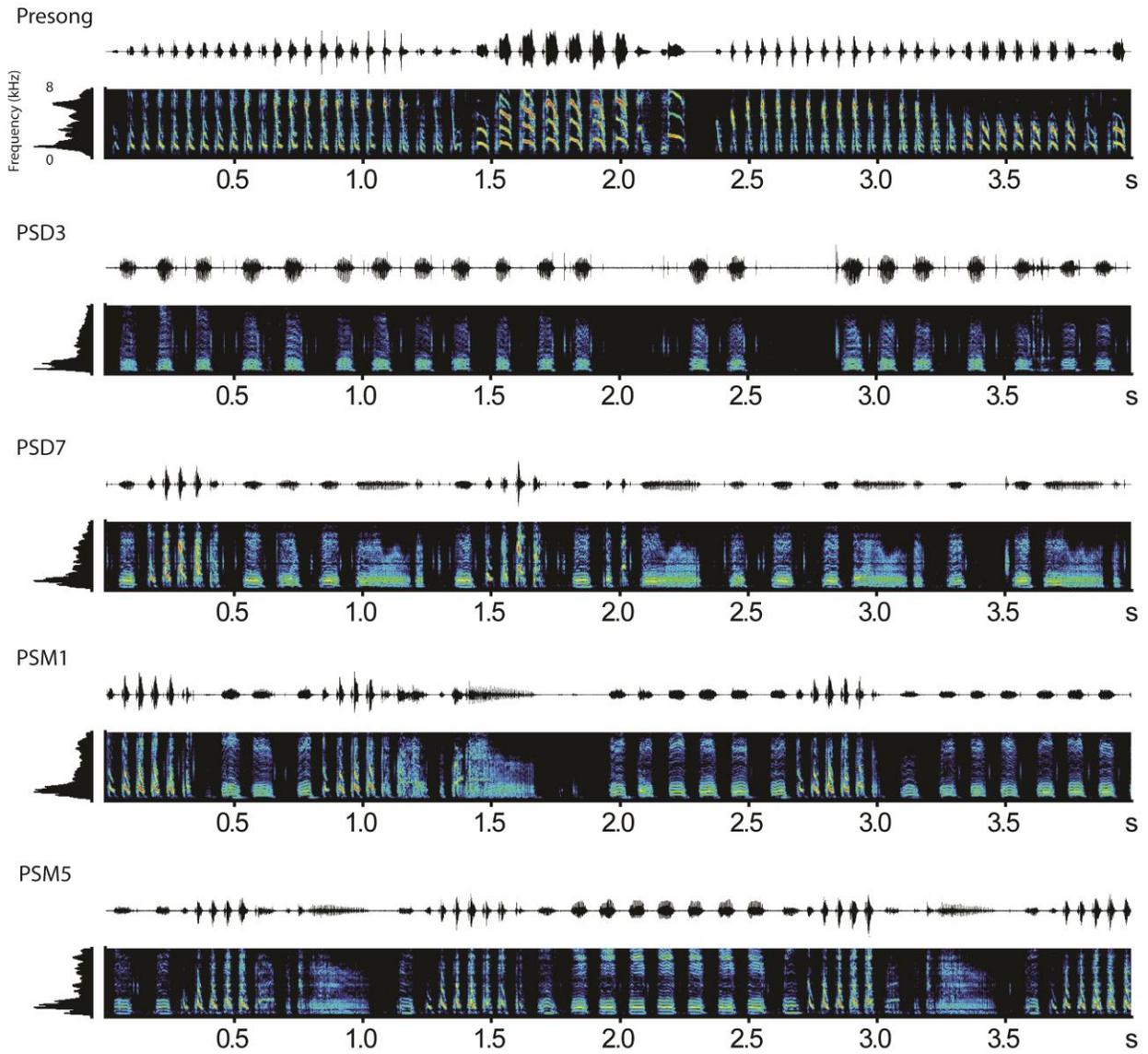


BFW37 Right HVC microlesion

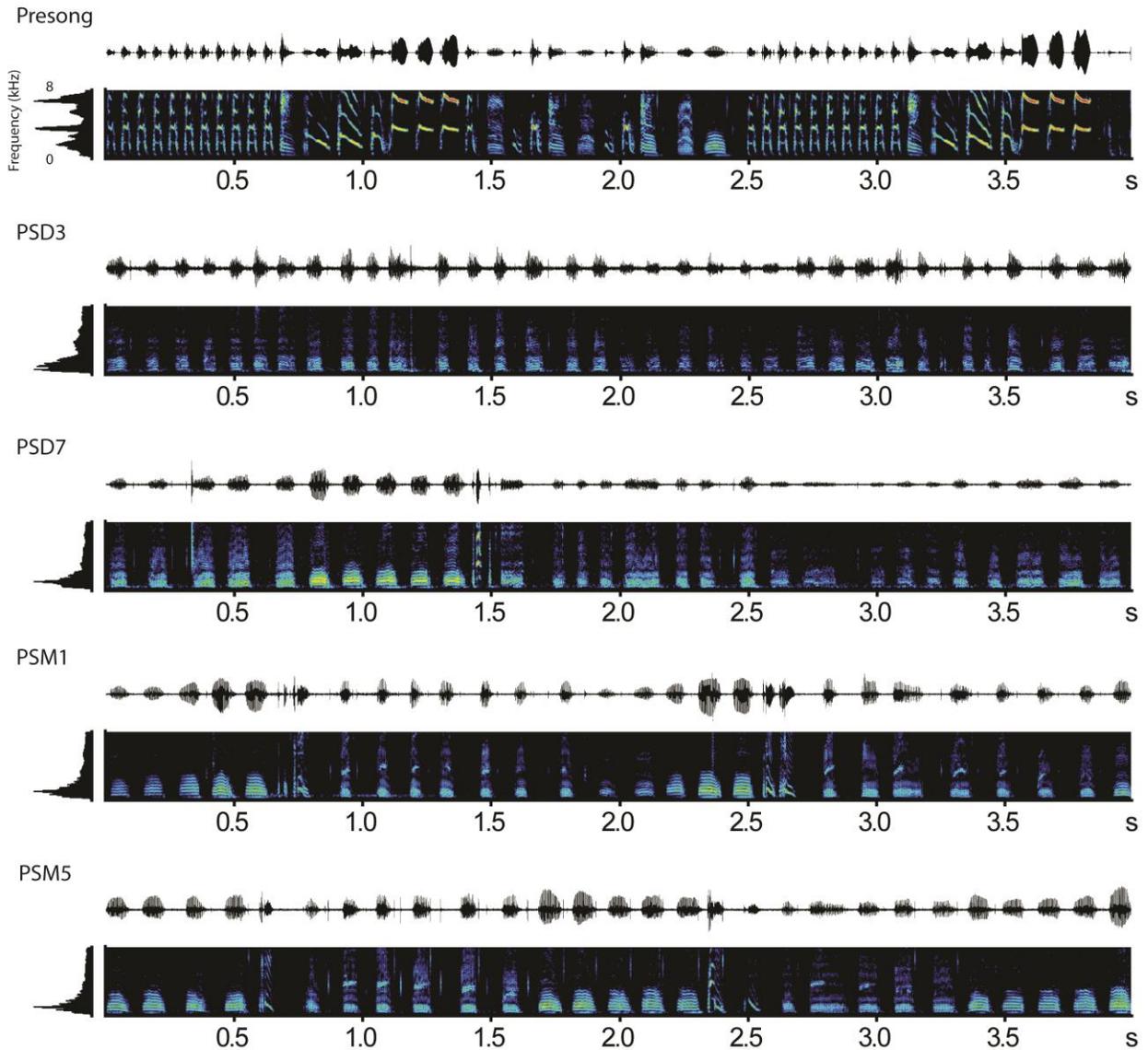


Appendix B: HVC full lesion song spectrograms

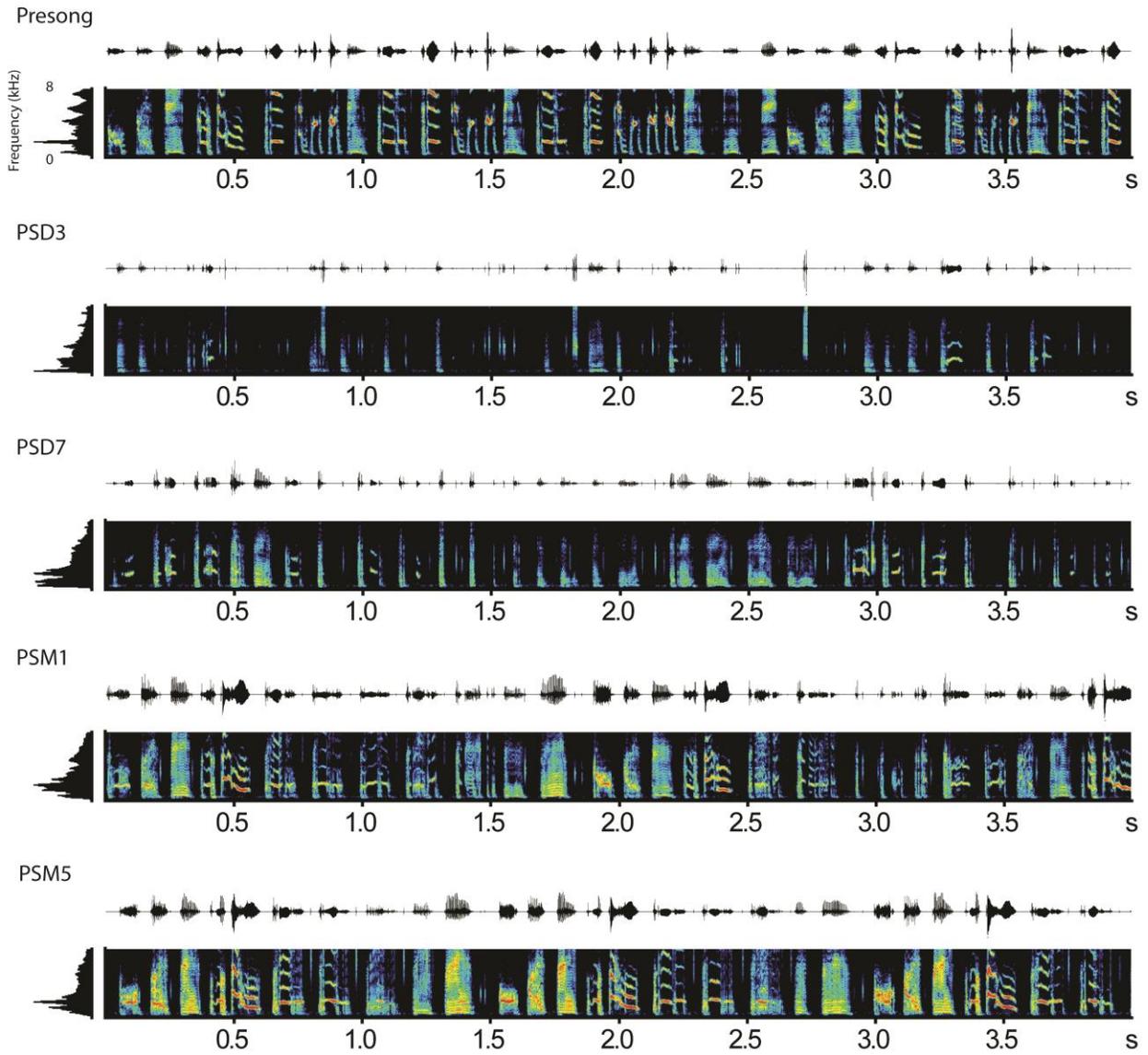
BFW50 Left HVC full lesion



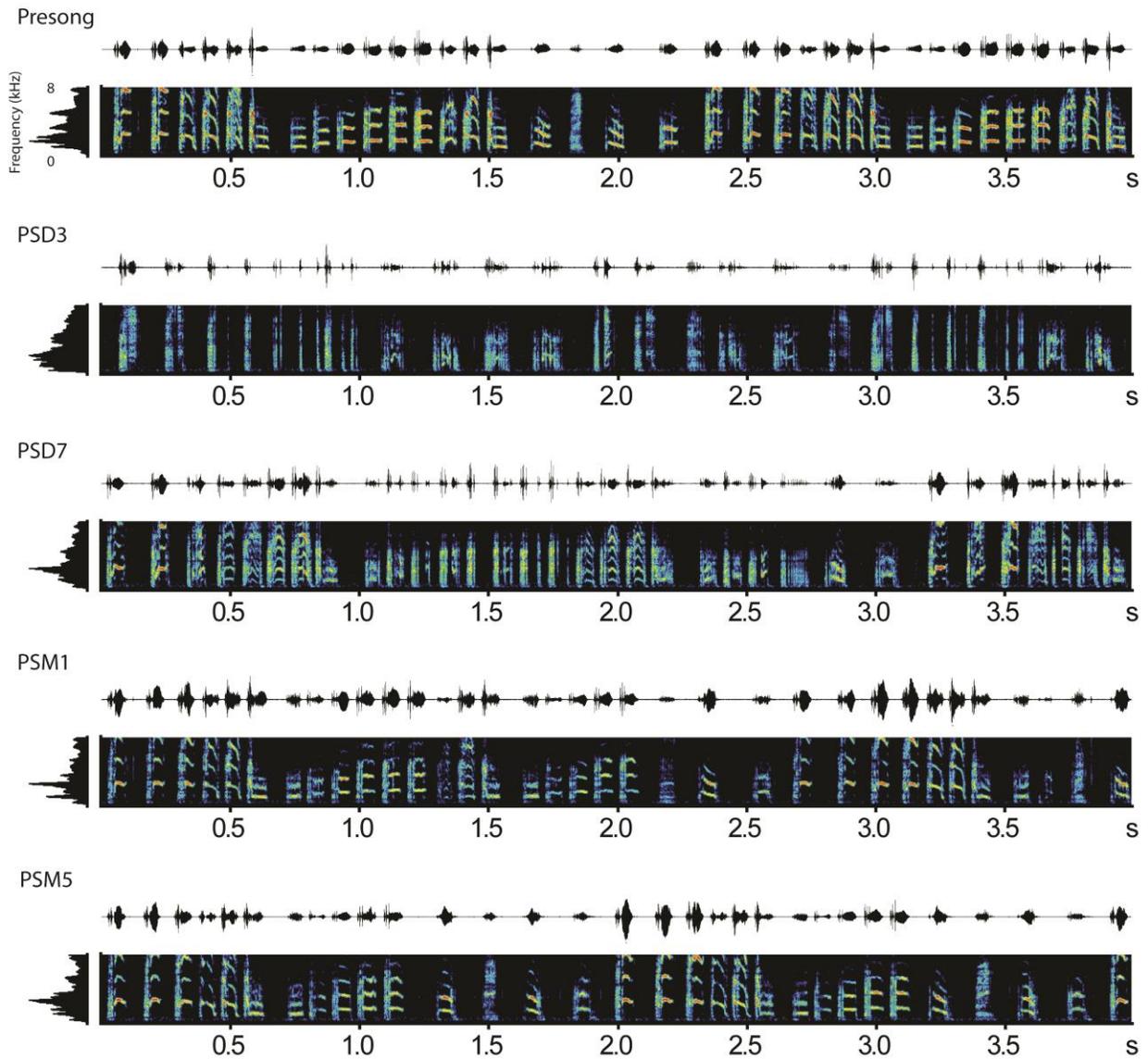
BFR100 Left HVC full lesion



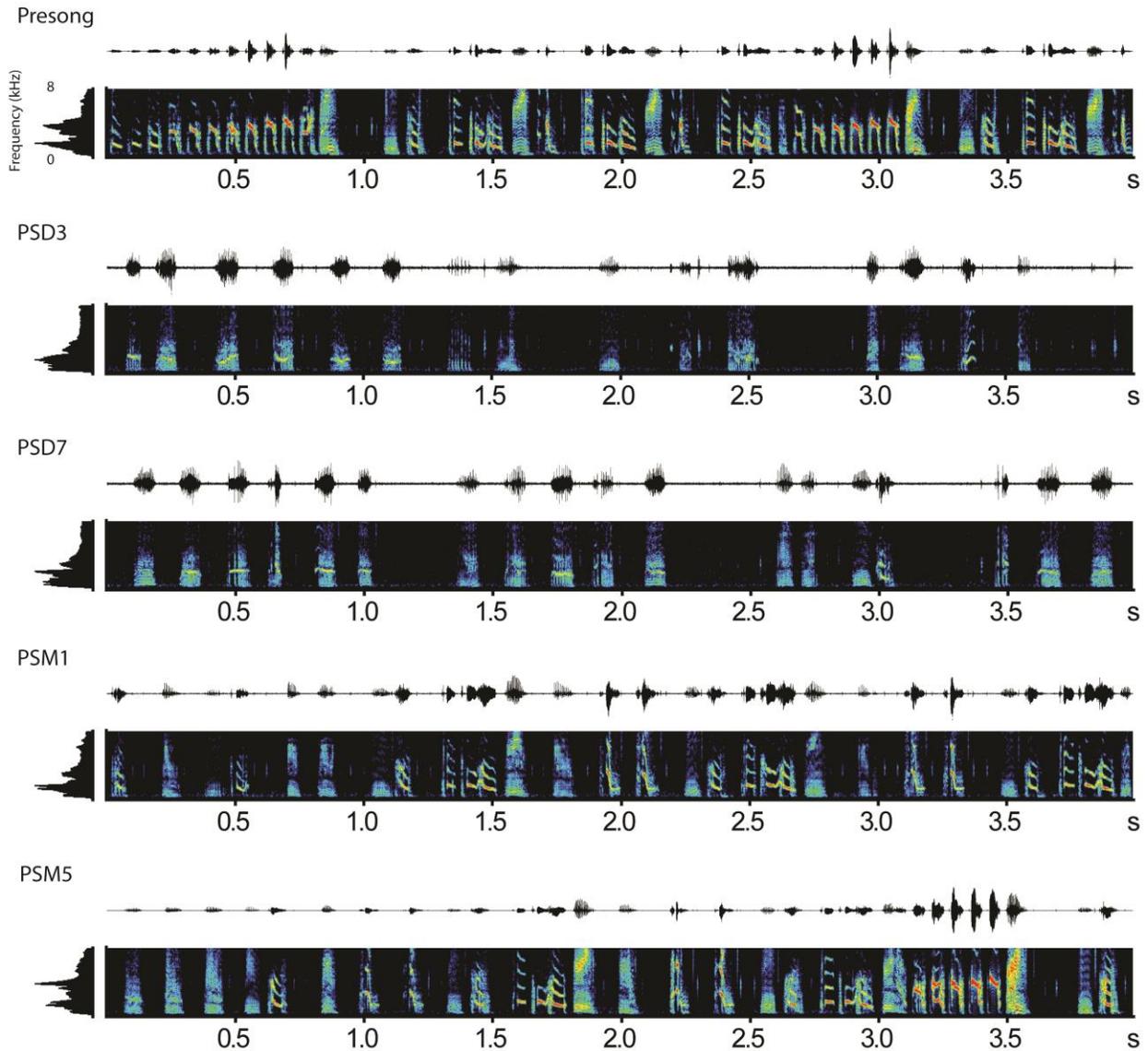
BFS683 Left HVC full lesion



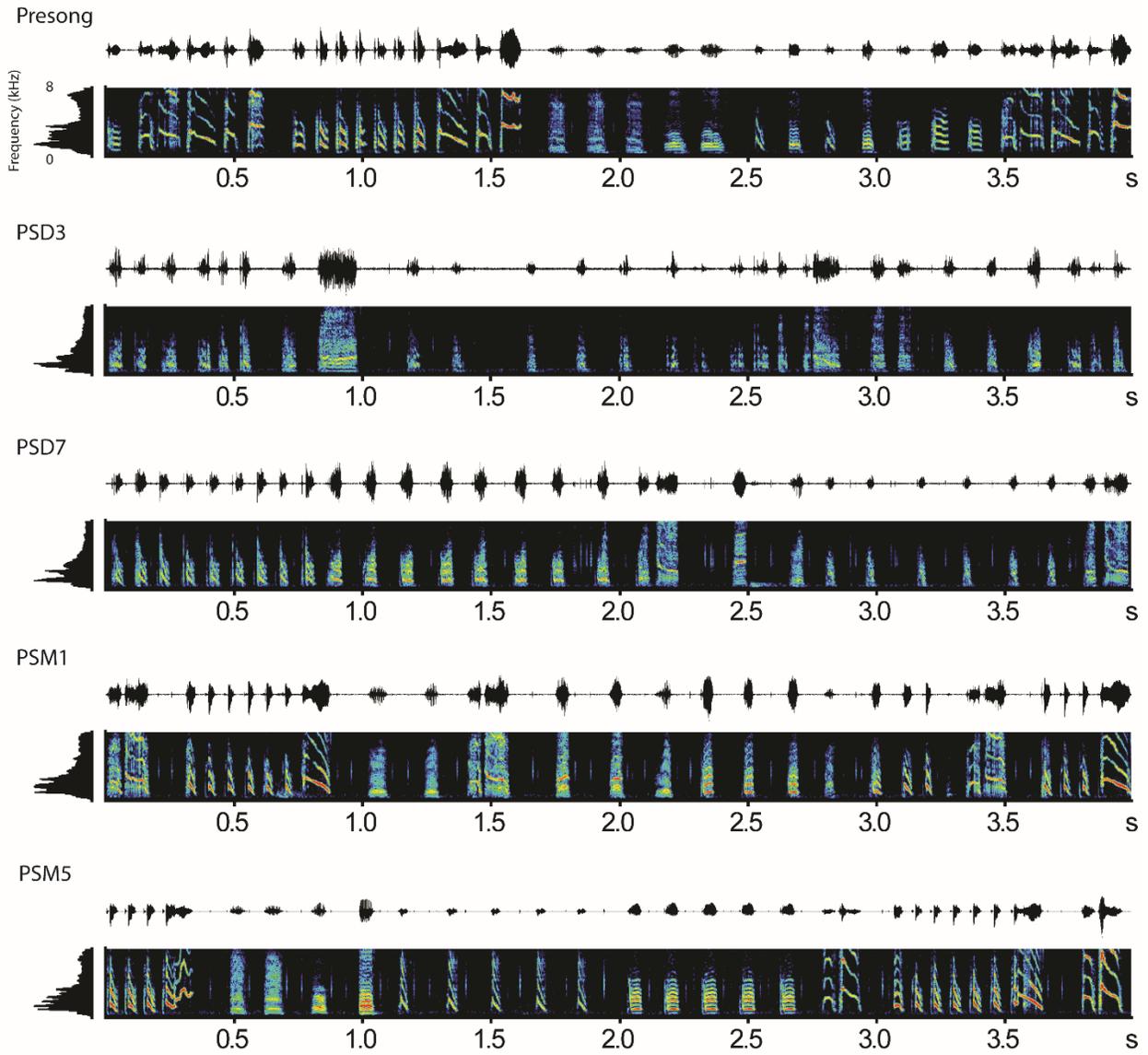
BFP4 Left HVC full lesion



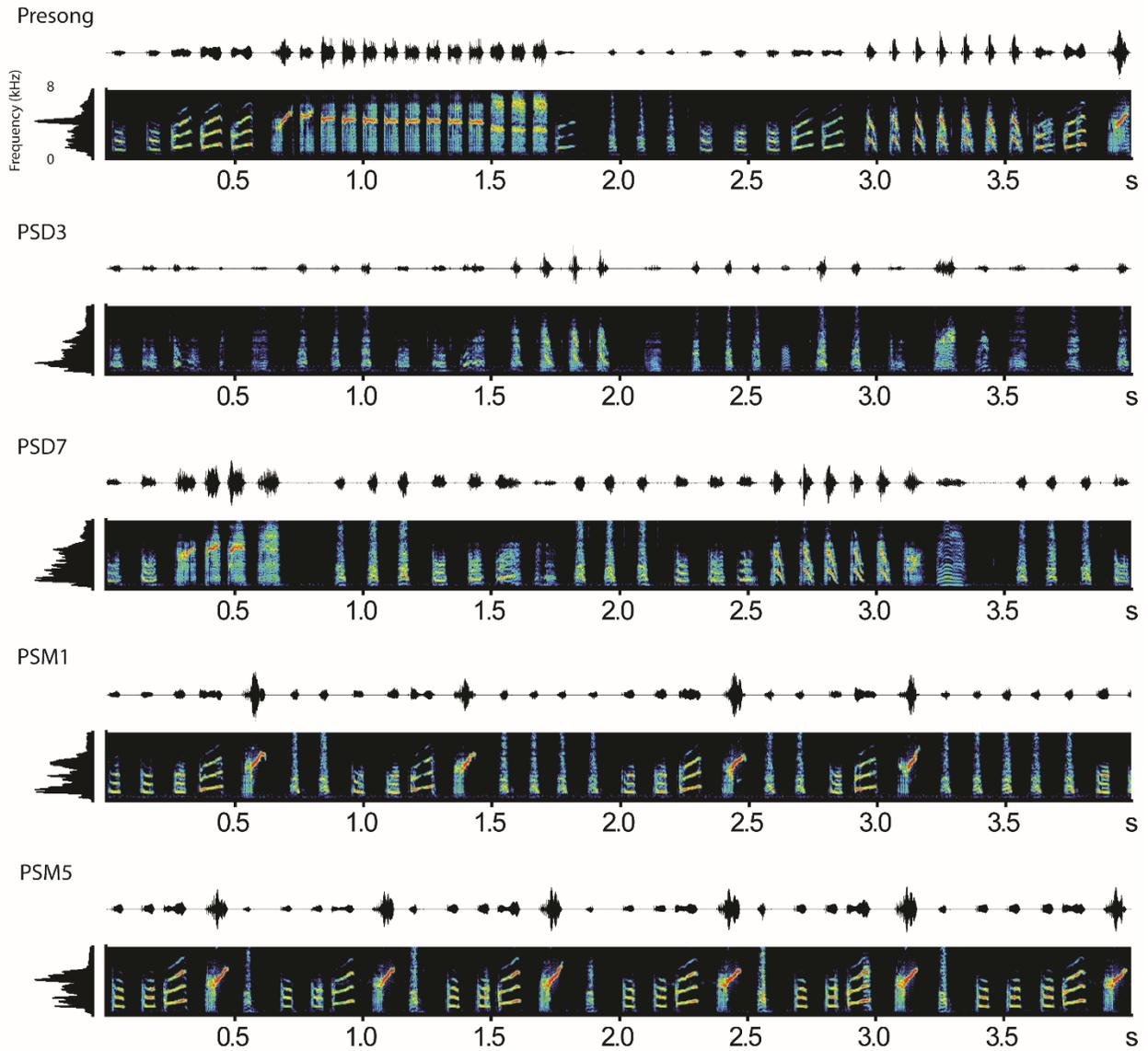
BFP45 Right HVC full lesion



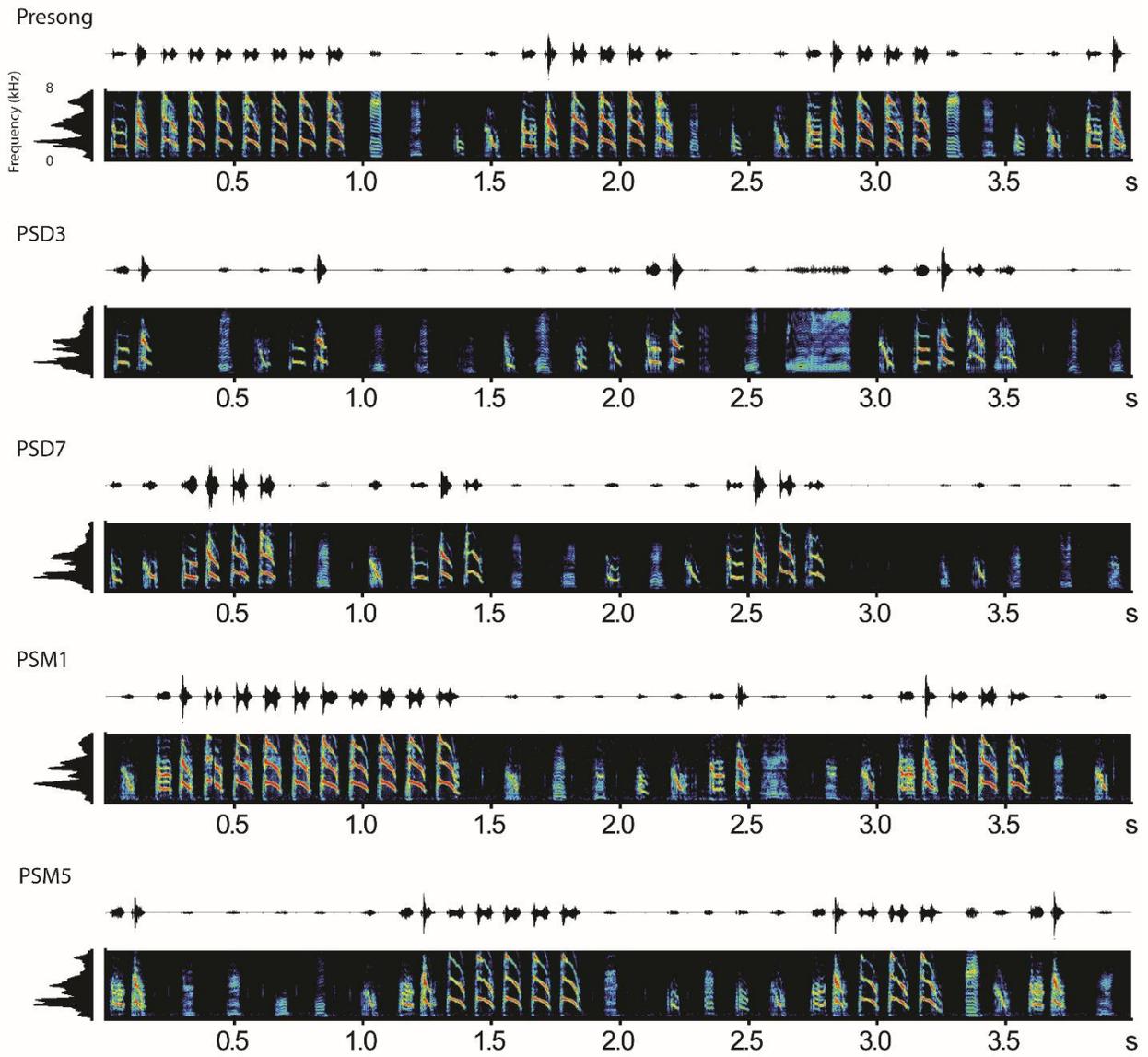
BFR63 Right HVC full lesion



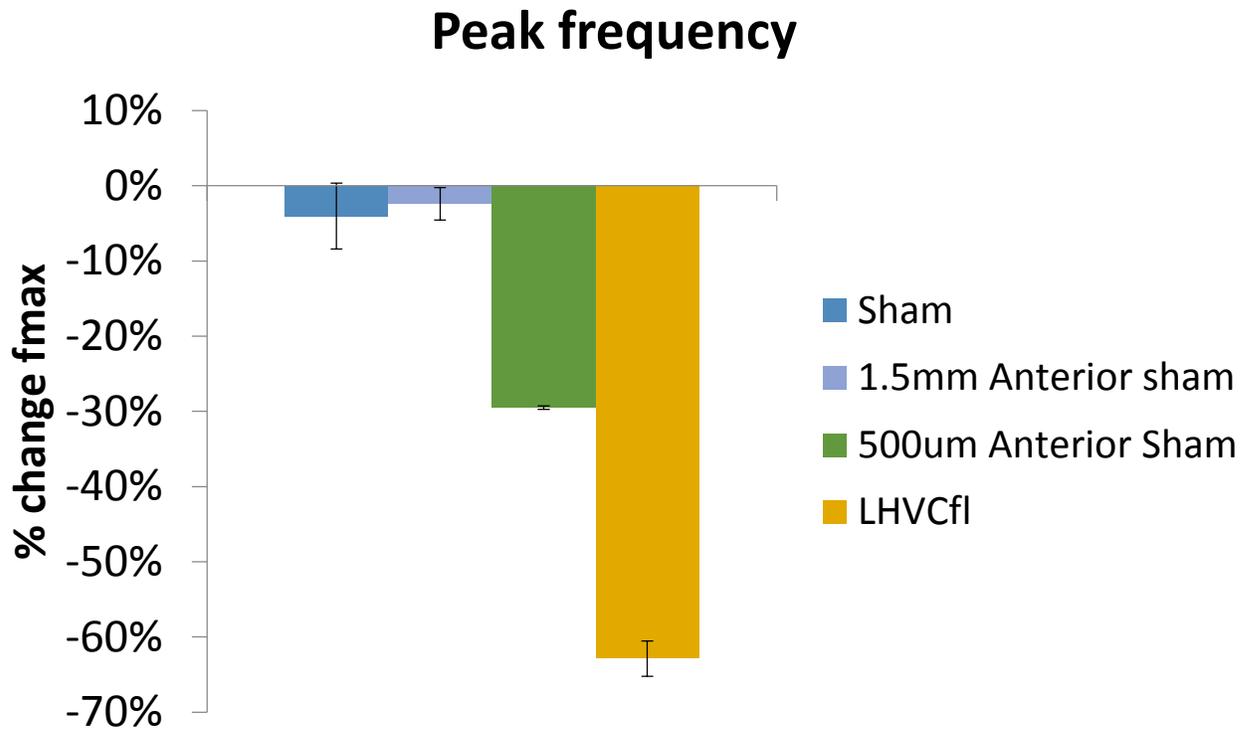
BFR77 Right HVC full lesion



BFW98 Right HVC full lesion

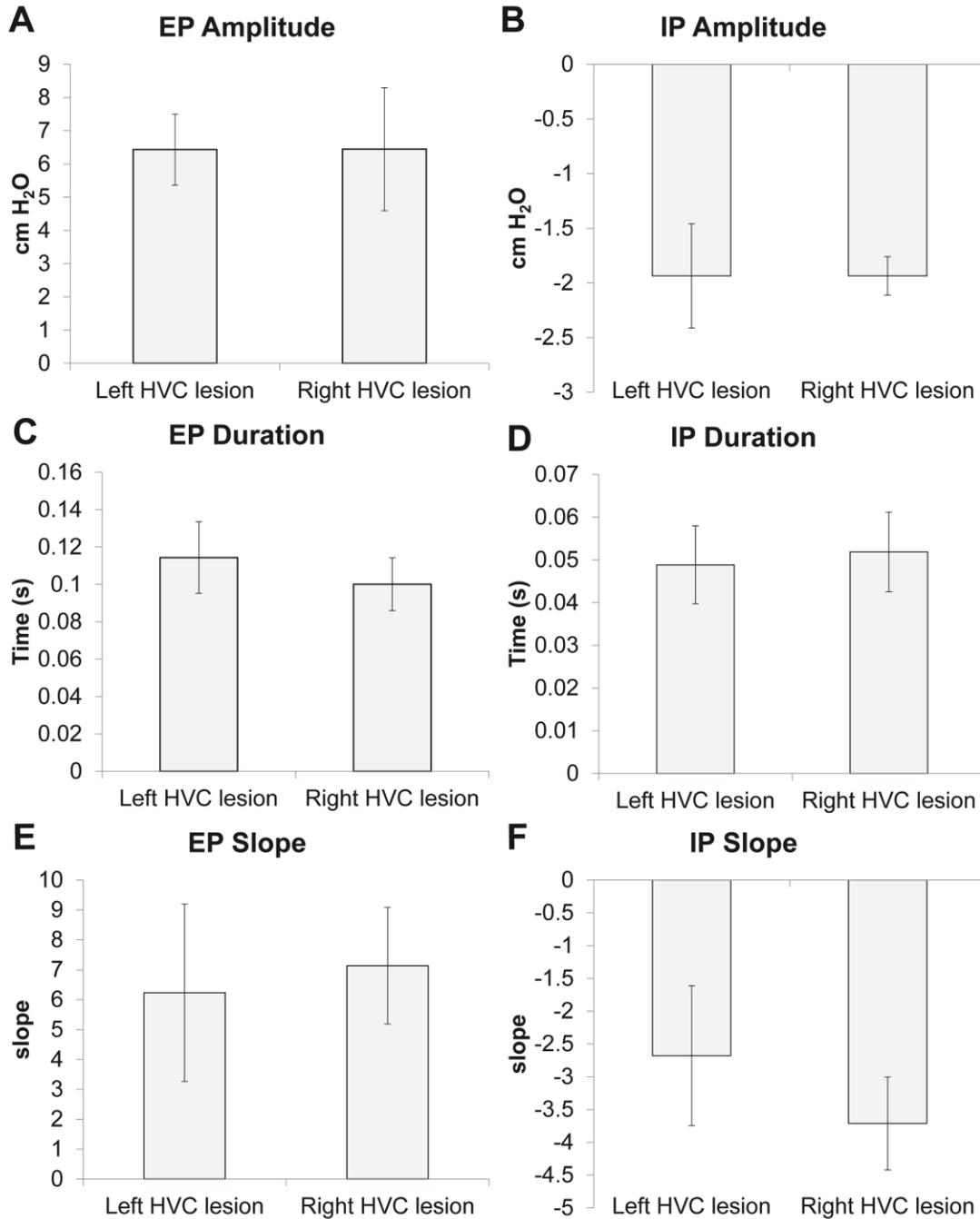


Appendix C: Location shams illustrate HVC specificity



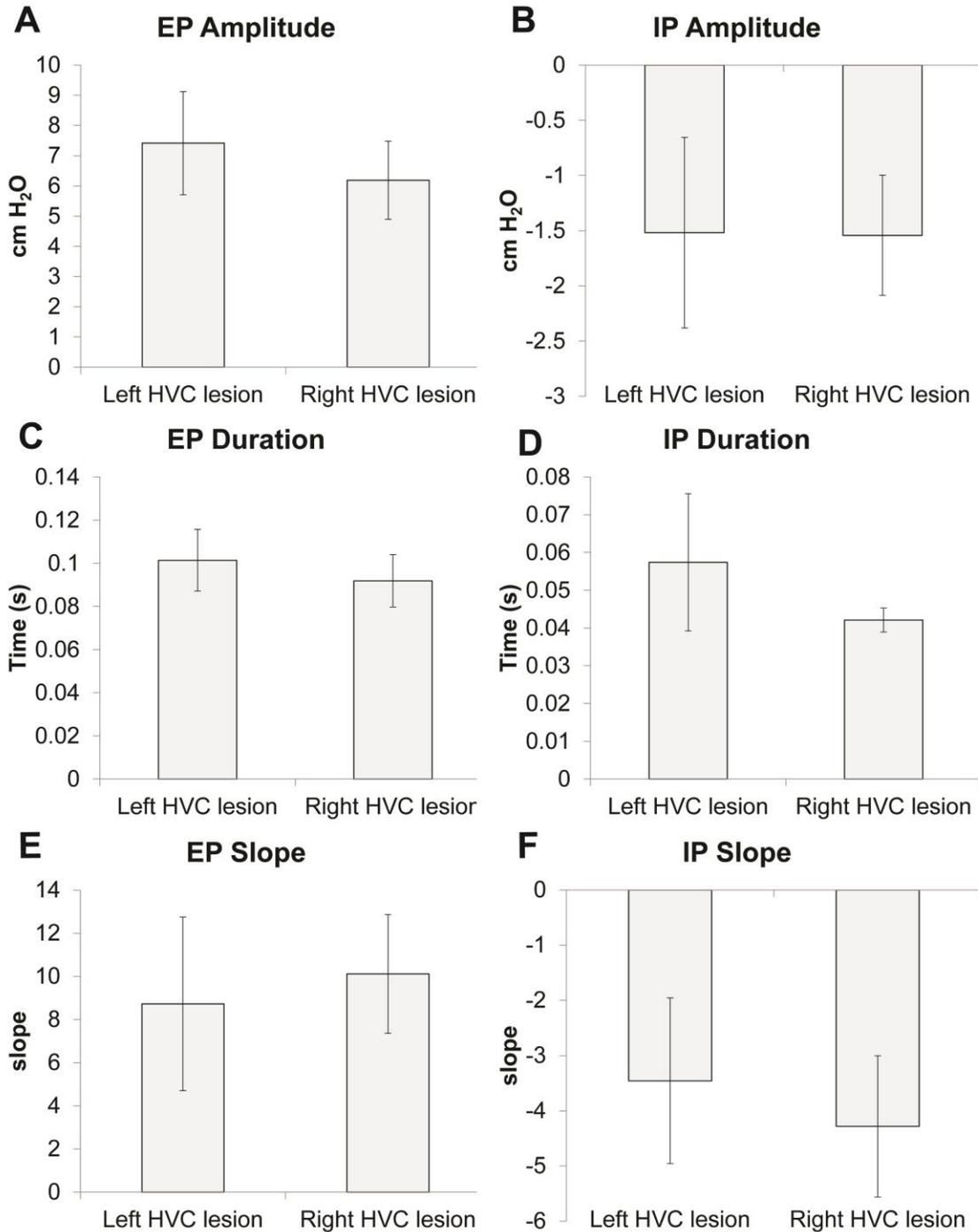
Location shams were performed by advancing the electrode 500 μm and 1.5 mm from the center of HVC and replicating the full lesion technique (briefly: 6 lesion sites, 100 μA current for 60 s).

Appendix D: Left and right HVC lesions produce similar effects on song respiratory features at the post-surgery timepoint



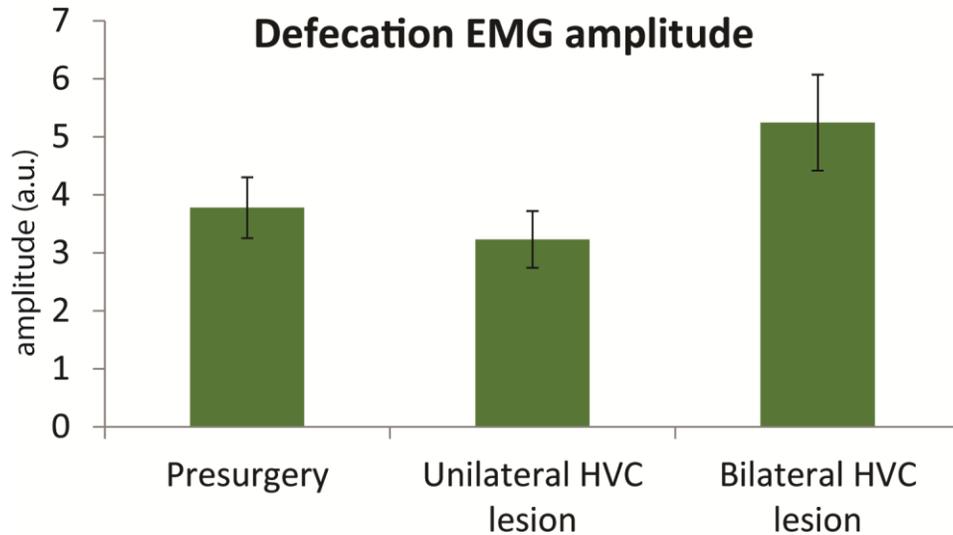
p > 0.1 on all features, unpaired t-test

Appendix E: Absence of left and right HVC lesion differences on song respiratory features at PSM6-8



p > 0.1 on all features, unpaired t-test

Appendix F: Expiratory EMG associated with non-vocalized behavior



Using defecation as a control confirms the observed reduction in post-lesion EMG amplitude was due to HVC damage and not signal decay. Defecation requires maximal expiratory activity and closing the bronchial lumen and was ideal as a consistent, comparable behavior.

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Vita

Personal Background

Catherine May Mancenido Urbano

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She has one brother

Education

- | | | |
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| 2009 | B.S. | Psychology (minor: Communication Sciences and Disorders)
Texas Christian University (Fort Worth, TX) |
| 2013 | M.S. | Experimental Psychology
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Thesis Title: Exploring peripheral lateralization in the Bengalese finch, <i>Lonchura striata domestica</i> |
| 2017 | Ph.D. | Experimental Psychology
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Professional Memberships

- | | |
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| Member, Society for Neuroscience | 2011 - present |
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Abstract

THE ROLE OF HVC IN COORDINATING ANATOMICALLY ASYMMETRIC SOUND SOURCES WITH A BILATERALLY CONTROLLED RESPIRATORY SYSTEM

By Catherine May Mancenido Urbano, PhD, 2017
Department of Psychology
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Dissertation Advisor: Brenton G. Cooper, Associate Professor of Psychology

Bengalese finch (*Lonchura striata domestica*) song is a learned behavior that is produced with remarkable asymmetry in spectral and amplitude control of sound production. Louder, higher frequency (>2.2 kHz) notes are generated by the left side of the syrinx (avian vocal organ) whereas softer, lower frequency notes are right-side generated (Urbano, 2013). We compared changes in song features and rate of recovery following left or right HVC microlesions or large (“full”) HVC lesions. Song was recorded from birds prior to, and for the first week following unilateral damage to HVC. Left and right HVC lesions lowered peak frequency and amplitude and increased the intersyllable interval (ISI), compared to sham lesions. The song deterioration induced by the microlesion was transient and birds largely recovered within one week. We continued to record full lesion groups for five months. We found evidence that Bengalese finches rely on left HVC for retention of higher frequency syllables. We also found evidence that left HVC damage leads to slower rates of short-term (within the first week) and long-term (over five months) recovery. Symmetric evidence of amplitude and temporal changes led us to investigate the effects of HVC lesion and inactivation on the underlying song respiratory pattern. Across several experiments, we provide direct evidence for how forebrain signals are translated into sound. In experiment 1, we presented data

illustrating the relationship between unilateral HVC lesion and resultant reduced song amplitude. Experiment 2 illustrated that amplitude will gradually recover months after large HVC lesion. Experiment 3 shed light on why songs are sung more quietly: the underlying reduction in EP amplitude and slope that, interestingly, does not recover months after surgery. Experiments 4 and 5 looked at potential peripheral mechanisms for the altered respiratory pattern and demonstrated that reducing HVC input (via lesion or inactivation) does two things: directly attenuates muscle activation and decouples the onset of dTB and EXP muscles. Studies 1 and 2 point establish the Bengalese finch as a potential animal model for studying the evolution of lateralized motor behavior. Studies 3-5 extend scientific understanding of phonation and the importance of coordinating vocal motor and respiratory motor systems for producing audible vocalizations.