

Effect of LMAN on learning of introductory vocalizations produced before the song by male zebra finch

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by

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Certificate

This is to certify that this dissertation entitled “**Effect of LMAN on learning of introductory vocalizations produced before the song by male zebra finch**” towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by **Anand C Krishnan** at Indian Institute of Science Education and Research, under the supervision of Dr Raghav Rajan, Assistant Professor, Department of Biology, during the academic year 2023-2024.



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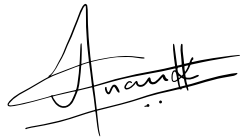
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This thesis is dedicated to *juvenile zebra finches*

Declaration

I hereby declare that the matter embodied in the report entitled “Effect of LMAN on learning of introductory vocalizations produced before the song by male zebra finch” are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education & Research (IISER) Pune, under the supervision of Dr Raghav Rajan, and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgment of collaborative research and discussions. This is a collaborative project with Vidushi Sharma and Shikha Kalra.

A handwritten signature in black ink, appearing to read 'Anand C Krishnan', with a stylized flourish at the end.

Anand C Krishnan

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Table of Contents

<i>Abstract</i>	10
<i>Acknowledgments</i>	11
<i>Contributions</i>	14
CHAPTER 1: Introduction	15
1.1 Learning through mimicking	16
1.2 Songbirds: good model organism to study vocal learning.....	16
1.3 Song of zebra finches.....	17
1.4 Song learning in zebra finches	18
1.5 The song pathway.....	19
1.5.1 AFP is important for song learning.....	20
1.5.2 AFP generates variability in learned vocalizations	22
1.6 INs can also be learned from a tutor	23
1.7 Does LMAN affect learning of INs?	23
CHAPTER 2: Materials and Methods	26
2.1 Experimental birds.....	27
2.2 Experimental design.....	27
2.3 Tutors.....	29
2.4 Song Recordings	29
2.5 Surgical procedures.....	30
2.5.1 Antidromic stimulation	31
2.5.2 Bilateral electrolytic lesions.....	33
2.5.3 Tracer injection	33
2.6 Perfusion and tissue preparation	36
2.7 Histology.....	36
2.7.1 Immunohistochemistry.....	36

2.7.2	Cresyl violet staining	40
2.7.3	Quantification of LMAN remaining	42
2.7.4	Tracer	43
2.8	Data analysis	44
2.8.1	Song segmentation and syllable labeling	44
2.8.2	IN number calculation.....	44
2.8.3	IN acoustic structure	45
		46
CHAPTER 3: Results		46
3.1	Standardizing LMAN coordinates.....	47
3.1.1	Location of LMAN was identified using antidromic stimulation and tracer injection in RA	47
3.1.2	Standardization of lesion parameters	48
3.2	Visualized and quantified LMAN using cresyl violet staining	50
3.2.1	LMAN is observable in birds with sham lesions but not in birds with LMAN lesions following cresyl violet staining.....	50
3.3	Lesions of LMAN affects learning acoustic structure of INs and motifs	52
3.3.1	Song spectrogram of sham lesioned pupil versus its tutor	52
3.3.2	Song spectrogram of LMAN lesioned pupil versus its tutor	54
3.3.3	Motif similarity is much lower for LMAN lesioned birds versus socially tutored birds.....	54
3.3.4	Unilateral lesions of LMAN may also affect learning the acoustic structure of motif syllables ...	55
3.3.5	IN similarity is much lower for LMAN lesioned bird versus socially tutored bird.....	56
3.3.6	INs and Motifs are learned independently of each other	57
3.4	LMAN may influence learning of IN number	58
CHAPTER 4: Discussion		60
4.1	Standardized LMAN coordinates, lesion parameters, and staining method	61
4.2	LMAN influences learning the number and structure of INs	62
4.3	Songs of some lesioned pupils stereotyped after post hatch day 90	63
4.4	Some sham lesioned birds did not learn the tutor's song.....	63
	Future direction	66
References		67
Appendix		70

List of Tables

Table 2.1: Details of the antidromic stimulation surgeries performed.....	32
Table 2.2: Details of the bilateral LMAN lesion surgeries performed on juvenile zebra finches	34
Table 2.3: Details of the bilateral LMAN lesion surgeries performed on adult zebra finches .	34
Table 2.4: Details of the bilateral sham lesion surgeries performed on juvenile zebra finches	35
Table 2.5: Details of the retrograde tracer injection surgeries performed.	36
Table 3.1: LMAN coordinates	49

List of Figures

Figure 1.1: Spectrogram of a male zebra finch song.	17
Figure 1.2: Song learning phases in zebra finch	18
Figure 1.3: Progression of song learning in zebra finches.....	19
Figure 1.4: Sagittal section of zebra finch brain marking different brain regions involved in song learning.....	21
Figure 1.5: Schematic of expected outcomes of LMAN lesion.....	24
Figure 2.1: Spectrogram of adult male zebra finch song with extreme number of INs.....	28
Figure 2.2: Schematic representation of the experimental design.	29
Figure 2.3: Schematic representation of antidromic stimulation in RA to locate LMAN.	31
Figure 2.4: Schematic representation of retrograde injection into RA to fluorescently label LMAN.....	35
Figure 2.5: Immunohistochemistry (using protocol 1) of sagittal sections of zebra finch brain.	38
Figure 2.6: Immunohistochemistry (using protocol 2) of sagittal sections of zebra finch brain.	39
Figure 2.7: Cresyl violet stained sagittal sections of zebra finch brain using protocol 1.	41
Figure 2.8: Cresyl violet stained sagittal sections of zebra finch brain using protocol 2.	42
Figure 2.9: Cresyl violet stained sagittal sections of zebra finch brain using protocol 3.	42
Figure 2.10: Confocal images from retrograde tracer injected bird.....	43
Figure 3.1: Spontaneous firing activity recorded from RA	47
Figure 3.2: Sagittal brain sections (40 μ m thick) of zebra finch brain stained with cresyl violet acetate showing locations of stimulating electrode and recording electrode.....	48
Figure 3.3: Sagittal brain sections (40 μ m thick) of zebra finch brain stained with cresyl violet acetate comparing the size of LMAN and lesion.....	49

Figure 3.4: Sagittal brain sections (40 μ m thick) of zebra finch brain stained with cresyl violet acetate showing LMAN and lesion.....	50
Figure 3.5: Sagittal brain sections (40 μ m thick) of zebra finch brain stained with cresyl violet acetate showing LMAN and lesion with the new coordinates.....	50
Figure 3.6: Sagittal brain sections (40 μ m thick) of LMAN lesioned zebra finch brain stained with cresyl violet acetate.....	51
Figure 3.7: Sagittal brain sections (40 μ m thick) of sham lesioned zebra finch brain stained with cresyl violet acetate.....	52
Figure 3.8: Volume of LMAN remaining	53
Figure 3.9: Song spectrogram of a sham lesioned pupil and its tutor.....	53
Figure 3.10: Song spectrogram of LMAN lesioned pupil and its tutor	54
Figure 3.11: Motif similarity of LMAN/sham lesioned and socially tutored birds.	55
Figure 3.12: Fraction of LMAN remaining in the left hemisphere versus the right hemisphere compared to the motif similarity between the tutor and the tutee.....	56
Figure 3.13: IN similarity of lesioned birds versus socially tutored birds.....	57
Figure 3.14: IN similarity versus motif similarity for lesioned birds.	58
Figure 3.15: Mean IN number for tutor versus pupil.....	59
Figure 4.1: Song spectrogram of LMAN lesioned tutee.....	64
Figure 4.2: Song spectrogram of sham lesioned tutee	64
Figure 4.3: Confocal images of LMAN showing projections to RA.....	65

Abstract

Performing motor actions like a tennis serve or a basketball free throw requires coordination between sensory inputs and motor outputs, which is learned through practice and experience through trial and error. Similarly, songbirds like zebra finch learn songs by listening and imitating their father's songs. Their song consists of repeats of a short-duration vocalization, 'introductory note' (IN), followed by a consistent sequence of syllables, 'motifs'. Song learning involves the sensory acquisition and the motor phase. Brain regions involved in the acquisition and production of songs include the anterior forebrain pathway (AFP) and motor pathway. AFP is a cortical-basal ganglia circuit with LMAN as its output brain region. Any disruption to AFP during the song learning phase affects the accuracy of motif copying. However, INs, similar to motifs, have both learned and innate components. Hence, any damage to LMAN in male juvenile zebra finch during the song learning phase may influence IN learning. I performed bilateral electrolytic LMAN lesions on juvenile zebra finches during the song learning phase to check this. Next, I quantified and compared the percentage similarity in IN and motif with the extent of LMAN lesioned. Further, I validated the lesion efficacy by quantifying the volume of LMAN remaining in cresyl violet stained sagittal brain sections. My results suggest that lesions of LMAN affect learning of the acoustic structure of both INs and motifs and the number of INs. However, it is difficult to draw firm conclusions due to the small number of birds.

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Contributions

Contributor name	Contributor role
Raghav Rajan, Shikha Kalra, Anand C Krishnan, Vidushi Sharma	Conceptualization Ideas
Raghav Rajan, Shikha Kalra, Anand C Krishnan, Vidushi Sharma	Methodology
Raghav Rajan, Shikha Kalra, Anand C Krishnan, Vidushi Sharma	Software
Raghav Rajan, Shikha Kalra, Anand C Krishnan, Vidushi Sharma	Validation
Raghav Rajan, Shikha Kalra, Anand C Krishnan, Vidushi Sharma	Formal analysis
Raghav Rajan, Anand C Krishnan, Vidushi Sharma	Investigation
Raghav Rajan	Resources
Anand C Krishnan, Vidushi Sharma	Data Curation
Anand C Krishnan	Writing - original draft preparation
Anand C Krishnan	Writing - review and editing
Anand C Krishnan	Visualization
Raghav Rajan	Supervision
Raghav Rajan	Project administration
Raghav Rajan	Funding acquisition

CHAPTER 1: Introduction

1.1 Learning through mimicking

Imitation learning is a way of acquiring a novel skill through observing and subsequently emulating a behavior from another individual who performs it. In this process, the learner needs to continuously adjust their motor output based on sensory inputs and the outcomes of their previous actions. It requires perceiving the behavior produced in the past, representing this information in the sensory system, and then modulating the motor output to replicate that sequence of behaviors (Ganos et al., 2012). Humans are very good at imitation learning, which is an important way to acquire motor skills. Consider the example of learning to play basketball: we strive to shoot the ball into the basket by imitating the techniques demonstrated by our coach. However, it requires several rounds of trial and error practice before we can truly master the skill. This is an example of imitation learning, where we acquire the skill by practicing and imitating the coach. Humans learn many motor skills, such as speech, writing, playing a musical instrument, or performing a sport by imitation and practice through trial and error. Additionally, it is a crucial method through which infants acquire a lot of motor skills from their parents or siblings.

1.2 Songbirds: good model organism to study vocal learning

Apart from humans, many animals utilize imitation to learn social skills. Indeed, imitation offers an efficient means for non-human species to gain skills and intellectual capacities that they might not have otherwise obtained, saving both time and energy (Byrne and Russon, 1998; Huber et al., 2009). Songbirds (passerine) belonging to the order Passeriformes are an excellent model organism to study imitation learning. These avian species communicate through vocalization, and they possess the capacity to learn and replicate diverse vocal patterns. Passerines are capable of learning songs, which are produced by a sequence of motor actions. They learn these songs through observation and imitation of fellow members of their species. More often than not, their vocal repertoire can be differentiated into calls and songs. The zebra finch, an Australian songbird (*Taeniopygia guttata*), is one of the most widely studied birds in this category. These birds are highly social, and they can survive in a wide range of habitats and environments (Zann 1996). Zebra finches are sexually dimorphic with differences in plumage on the throat, foreneck and breast (Zann 1996). Males of this species have orange cheek patches, black and white stripes on the throat and breast, and brown color on the sides

of the body. All these characteristics of zebra finches and their ability to learn a stereotyped song within 3-4 months post-hatching make them an excellent model organism for studying vocal learning.

1.3 Song of zebra finches

Only male individuals of this species have the ability to sing, and a typical bout of zebra finch song consists of introductory notes followed by motifs (Zann, 1996; Price, 1979). The vocalization that repeats multiple times at the beginning of a bout is called an introductory note or IN, and the stereotyped sequence of vocalizations that comes after the introductory notes is called a motif (Figure 1.1). A bout of zebra finch song may consist of multiple sets of INs and motifs separated by short pauses (500ms), and a bout is defined by a pause greater than 2s (Sossinka and Böhner, 1980). Zebra finch songs are broadly classified as directed or undirected songs based on social context (Zann 1996). If a male bird sings a song directed towards a female as part of their courtship display, it is called a directed song or courtship song. Alternatively, if a male bird sings a song when alone and not directed towards any particular individual, it is called an undirected song or solitary song (Immelmann 1969). The directed song produced by the male zebra finch, on average, has more number of INs, more motifs per bout, a faster tempo, and lesser variability in vocalizations when compared to the undirected song (Sossinka and Böhner, 1980; Kao et al., 2005).

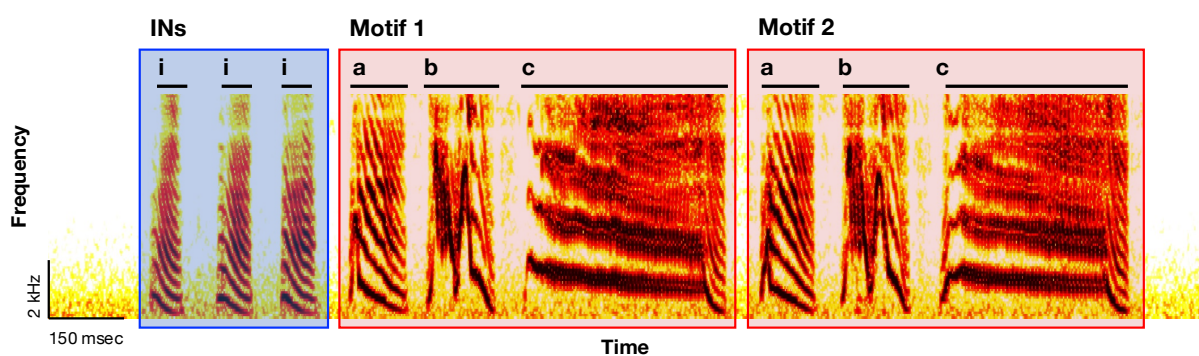


Figure 1.1: Spectrogram of a male zebra finch song.

The song can be differentiated into introductory notes (INs) and motifs. The vocalization repeating at the beginning of the song is called an IN (highlighted in blue), and the consistent sequence of vocalization that comes after the INs is called the motif (highlighted in red). The introductory notes are labeled as 'i', and the individual syllables in the motif are labeled as 'abc.' Scale bar: 150 msec (x-axis) and 2kHz (y-axis)

1.4 Song learning in zebra finches

There are a lot of parallels between birdsong and human speech. Similar to how human infants learn vocalization from their parents, songbirds learn their song from adults of their species. (Doupe and Kuhl, 1999). Male zebra finches learn songs very early in their life by listening and imitating the song of their father or a foster father (tutor). There are two overlapping phases in song learning (Immelmann, 1969; Price, 1979; Eales, 1985; Slater et al., 1988) (Figure 1.2). The first phase is called the sensory acquisition phase, where the male juvenile zebra finches listen to their father's song and memorize song elements from this template. The duration of this sensitive period is highly variable (Eales 1985). The experience or exposure to a song template determines the duration of the sensitive period and not the age of the bird. The later phase is called the sensory-motor phase when the juvenile bird babbles and practices the memorized vocalizations. During this time, these birds use their auditory feedback to improve their version of the song by fine-tuning the acoustic and temporal properties to match it with the memorized song template.

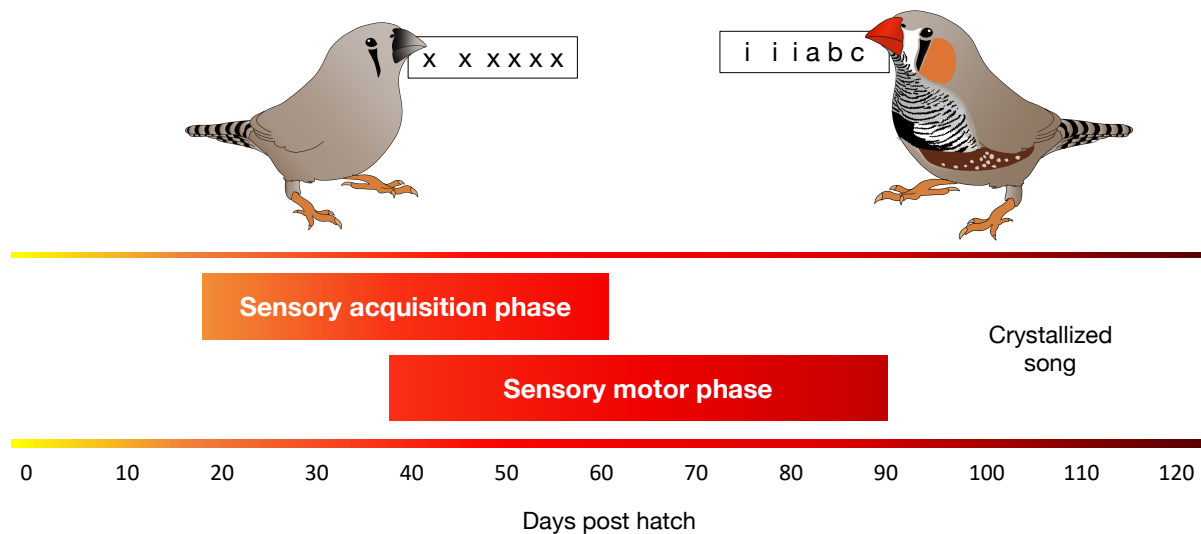


Figure 1.2: Song learning phases in zebra finch

Song learning consists of the sensory acquisition phase and the sensory motor phase. The juvenile bird (black beak) memorizes the father's song during the sensory acquisition phase. It tries to produce vocalization similar to the father's during the sensory motor phase. Father's song is represented by 'i i i a b c'. 'x x x x x x' represents unrecognizable vocalizations produced by the juvenile during the sensory motor phase. (Immelmann, 1969 and Eales, 1985)

During the sensory-motor phase, these juvenile birds begin with a highly variable song without any recognizable syllables, called a "*subsong*" (Figure 1.3 A). The acoustic structure and the

duration of syllable/inter-syllable intervals are variable for the subsong, and it looks very different from the song template of the father or the tutor. Over the course of the next few days, the juveniles progress to produce a “*plastic song*” with some syllables similar to the template song (Figure 1.3 B). Ultimately, around 90 days after hatching, they acquire the ability to produce a stereotyped song, which can be differentiated into INs and motifs (Figure 1.3 C). All male juvenile zebra finches go through these phases of learning, but the timing and duration of each phase can be highly variable.

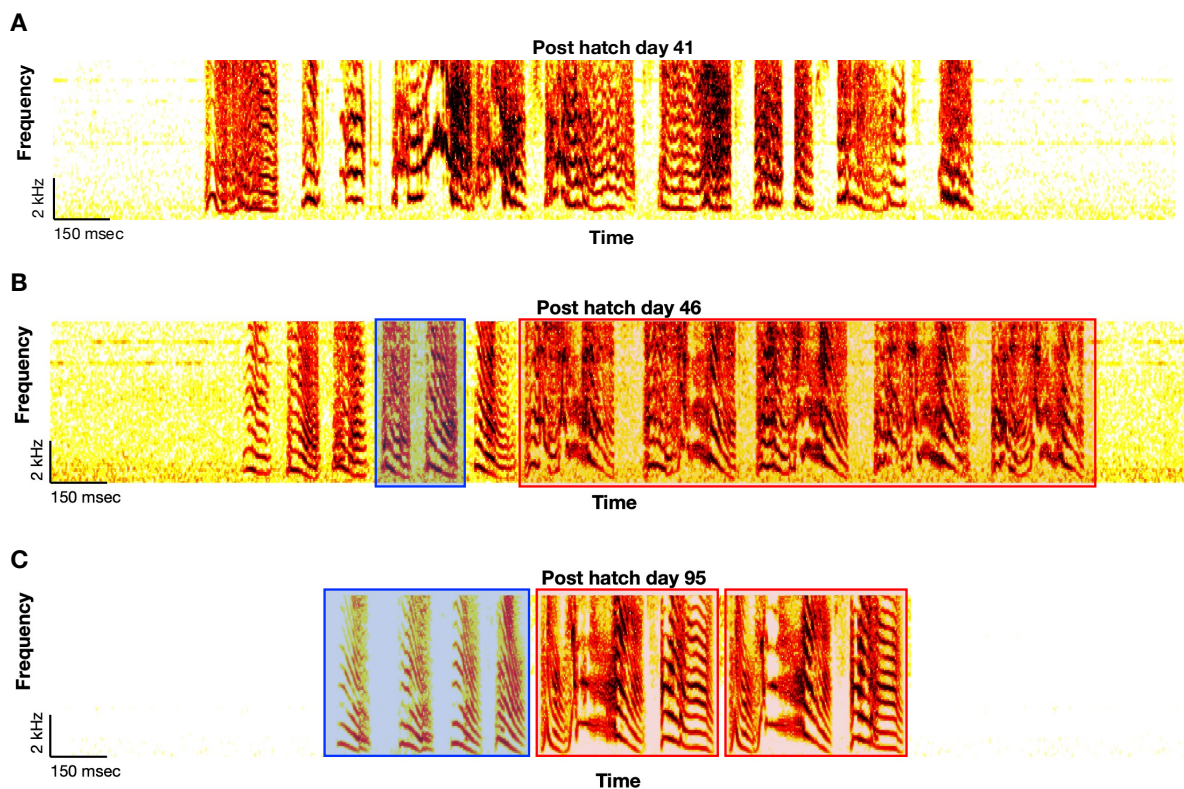


Figure 1.3: Progression of song learning in zebra finches.

Spectrograms of song produced by the same bird during song learning phases. A) ‘subsong’ produced by the juvenile (recorded on post-hatch day 41), where there is no distinguishable syllable. Juvenile finches gradually transition from subsong to a ‘plastic song’ (B). The syllables highlighted in blue and red represent two distinct sets of recognizable syllables produced by the juvenile. C) shows the final song produced by the bird. This final song is highly stereotyped, with vocalizations distinguishable into introductory notes (INs) highlighted in blue and motifs highlighted in red. The song recordings utilized to generate these graphs were provided by Shikha Kalra. Scale bar in (A), (B), and (C): 150 msec (x-axis) and 2kHz (y-axis)

1.5 The song pathway

The neural circuits involved in the learning and production of songs have been studied extensively. In 1976, Nottebohm et al. conducted one of the initial investigations, employing a

range of behavioral and anatomical methods to identify the neural pathways responsible for controlling song in canaries. They performed electrolytic lesions to different parts of the brain and looked at changes in song by analyzing the pre-lesion and post-lesion songs from the birds. The results from their lesion experiments showed that even partial lesions of HVC (used as the proper name) affected the syllable number and structure in the postoperative song (Nottebohm et al., 1976). Bilateral lesions of HVC resulted in a notable effect on the song. Despite displaying motivation for singing, as evidenced by courtship posture and related movements, these birds experienced significant disruptions in motor movements, resulting in silent song (Nottebohm et al., 1976). This included notable impacts on the movements of the bill, larynx, syrinx, and respiratory muscles. By tracking down the degenerating fibers from HVC, they found two other discrete nuclei in the forebrain called RA (robust nucleus of the arcopallium) and Area X. Even though Area X lesions did not significantly affect song, RA lesions made the birds mute (Nottebohm et al., 1976). HVC projects to RA, and this pathway, which is essential for producing learned vocalization, is called the song motor pathway (Figure 1.4, shown in red). RA further projects to the downstream thalamic region and finally to the syringeal and respiratory muscles that must be precisely controlled to produce the vocalizations. HVC also projects to another forebrain nucleus called Area X, which indirectly projects to RA. Area X has projections to a thalamic region called the DLM (medial portion of the dorsolateral thalamus), which projects to LMAN (lateral magnocellular nucleus of the anterior nidopallium), and further LMAN projects to RA. This indirect projection from HVC to RA via Area X, DLM, and LMAN is called the anterior forebrain pathway (AFP) (Figure 1.4, shown in blue) (Bottjer et al., 1984). There are a lot of similarities between the mammalian cortex-basal ganglia-thalamocortical loop and the AFP in zebra finches (Gale and Perkel, 2010). LMAN is a cortical nucleus, DLM is part of the thalamus, and Area X in songbirds is the basal ganglia portion of the AFP. The various cell types in Area X and their anatomical communications to the different cortical nuclei like HVC, RA, and LMAN in songbirds are similar to that seen in the mammalian basal ganglia circuit and how it interacts with the other cortical regions (Fee and Scharff, 2010).

1.5.1 AFP is important for song learning

Bottjer et al. demonstrated for the first time in 1984 that a lesion to a specific brain area during a restricted developmental period could cause a permanent impairment to a learned motor

sequence. LMAN is the output nucleus of the AFP, and hence, lesioning LMAN would disrupt all the inputs from the AFP to RA in the motor pathway. Lesions of LMAN disrupted song learning in juvenile birds; however, it did not have any notable impact on the vocalizations that had already been learned by adult birds (Scharff and Nottebohm, 1991; Bottjer et al., 1984). Subsequent research conducted by Scharff and Nottebohm in 1991 compared the effects of lesioning different nuclei in the AFP and that of early life deafening on learning songs in juvenile zebra finches. By performing electrolytic bilateral lesions of Area X and LMAN in distinct groups of male juvenile zebra finches, Scharff and Nottebohm discovered that these two nuclei exerted different effects on song development. Lesions in Area X led to the production of unusually long and variable vocalizations in the birds, resulting in a prolonged period for their songs to crystallize, but it did not have any remarkable effect on the production of learned vocalization by adults (Scharff and Nottebohm, 1991; Sohrabji et al., 1990). In contrast, birds with lesions in LMAN achieved stereotypy in their songs earlier, and their songs were characterized by monotonous repetitions of fewer syllables (Scharff and Nottebohm, 1991). Thus, AFP plays a vital role in vocal plasticity, which is essential for learning (Scharff and Nottebohm, 1991; Brainard, 2004).

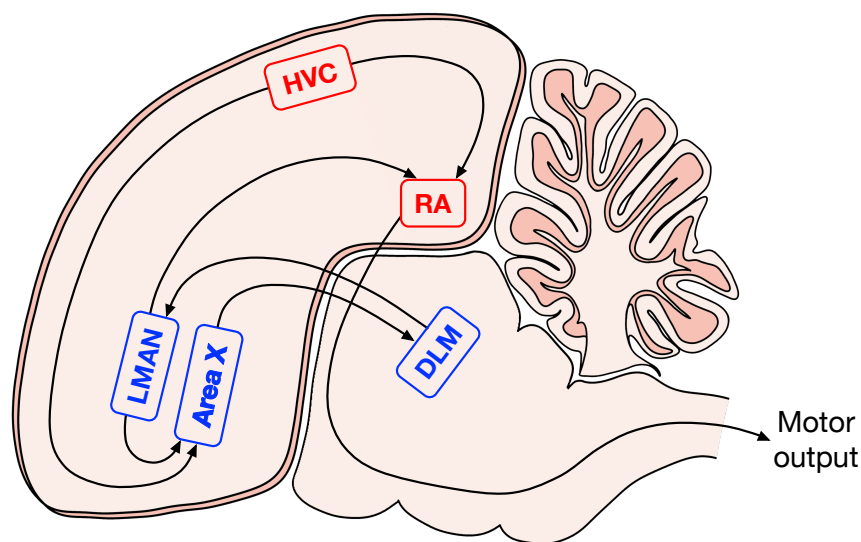


Figure 1.4: Sagittal section of zebra finch brain marking different brain regions involved in song learning. The motor pathway (red) includes HVC (used as the proper name) and RA (robust nucleus of the arcopallium), which are necessary for the production of learned vocalization throughout life. The anterior forebrain pathway or the AFP (blue) includes Area X, LMAN (lateral magnocellular nucleus of the anterior nidopallium), and DLM (medial portion of the dorsolateral thalamus). The AFP is necessary for song learning in juveniles and for vocal motor plasticity in adult birds but not for producing learned vocalizations (Nottebohm et al., 1976; Bottjer et al., 1984).

1.5.2 AFP generates variability in learned vocalizations

In addition to the normal progression of song learning in juvenile finches, the AFP also plays a critical role in the experience-dependent plasticity of adult zebra finch songs (Brainard 2004). Disruption of signals from the AFP influences certain aspects of the adult bird's song, though not every characteristic is affected. Experiments by Kao and Brainard in 2006 on the output nuclei of AFP (LMAN) show that such lesions prevent social context-dependent variability in the syllable structure of adult zebra finch songs. It reduces the variability in the syllables of an undirected song to a level seen in the directed song. Young adults had a broader distribution for the fundamental frequency of vocalizations in the undirected song compared to the directed song, but this broader distribution disappeared with LMAN lesions (Kao and Brainard, 2006). These effects of the lesions are evident as early as 2-3 days post-surgery and persist for an extended duration (Kao and Brainard, 2006). On the other hand, LMAN lesions do not affect some features of the song, like the number of introductory notes, the number of motif repetitions in a bout, the ordering of syllables, or the motivational aspect of courtship (Kao and Brainard, 2006). Electrolytic lesions of LMAN appear to affect the social regulation of song tempo (Kao and Brainard, 2006). However, subsequent research by Stepanek and Doupe (2010) demonstrates that the inactivation of LMAN by muscimol does not affect the social regulation of song tempo. Further, the singing-related activity of neurons in LMAN is more variable during undirected songs than during directed songs. Hence, this greater variability of the undirected song may originate from the increased variability of LMAN activity during undirected songs (Kao et al. 2005). All these findings provide evidence supporting the proposition that LMAN could play a part in the variability of songs dependent on social context.

Ölveczky et al. (2005) conducted experiments in juveniles where they inactivated LMAN using tetrodotoxin (TTX) or muscimol injections, demonstrating the significance of the inputs from the AFP to the motor system in vocal variability and song acquisition. Additionally, neural recordings from LMAN neurons projecting to the motor pathway showed differential spiking activity across multiple song renditions (Ölveczky et al., 2005). Stepanek and Doupe (2010) performed reversible inactivation of LMAN in awake singing adult zebra finches using muscimol, a GABA_A receptor agonist. Unlike lesions, these drug-induced changes do not damage the surrounding tissue, and the recovery period from inhibition is relatively brief. Such pharmacological inactivation also decreased the variability in the undirected song to a level

comparable to that observed in the directed song (Stepanek and Doupe, 2010). Furthermore, flushing out the drug from LMAN restored the variability in the undirected song to what it was before inactivation, confirming once more that LMAN activity can influence the variability in the song of adult zebra finches. (Stepanek and Doupe, 2010).

1.6 INs can also be learned from a tutor

Over recent decades, scientists have investigated the role of AFP and its output nucleus, LMAN, in learning of song in juvenile male zebra finches. However, previous research has predominantly focused on motifs within the songs rather than on INs, as most researchers thought that only motifs are learned and INs were viewed as innate. In 2021, Kalra et al. demonstrated through various tutoring methods that, akin to syllables within a motif, the introductory notes (INs) produced preceding the motif can also be acquired from a tutor. These experiments were done on juvenile zebra finches that were separated from their father at a very early age (to prevent any influence of the father's song on the juvenile), and they were tutored by a social tutor. The songs were recorded, and the similarity in INs was calculated between the tutor and the tutored birds after post-hatch day 90. Their findings indicated that these male juveniles can learn the average number of INs and their acoustic structure from a template song they were exposed to during the critical period for song learning (Kalra et al., 2021). At the same time, there are inherent biological predispositions influencing the learning and production of INs. Even when male juveniles were tutored with songs devoid of any INs or, in the case of isolates (where juvenile finches were visually isolated from other birds), they still integrated a few repetitive vocalizations at the beginning of the song that resembled INs. This provides evidence suggesting that INs can be influenced by learning experiences and inherent biological predispositions (Kalra et al., 2021).

1.7 Does LMAN affect learning of INs?

Suppose introductory notes can also be learned from a tutor in a way similar to other syllables in the motif. Then, LMAN might play a role in learning specific properties of INs, particularly their number and acoustic structure. At the same time, the innate properties of INs would remain unaffected even with lesions of LMAN. Therefore, my thesis aims to investigate the role of LMAN in learning the INs produced by male zebra finches.

A recent study from our lab has shown that specific properties of INs, like the number and acoustic structure, can be learned by juveniles, akin to how they learn songs. Given this context, if LMAN affects IN learning, then bilateral lesions of LMAN would hinder the juvenile birds from learning both INs and motifs from the tutor (Figure 1.5 [B]). Conversely, if LMAN does not affect IN learning, then I would expect that even with a complete bilateral lesion of LMAN, the juvenile bird would still learn and produce the introductory elements similar to the tutor, but it would not be able to learn the song from the tutor (Figure 1.5 [A]). Previous work by Shikha Kalra from our lab suggests that LMAN may have a role in learning the acoustic structure of INs from the tutor but not the number (Kalra, 2023). However, the number of birds with bilateral lesions was insufficient to draw firm conclusions.

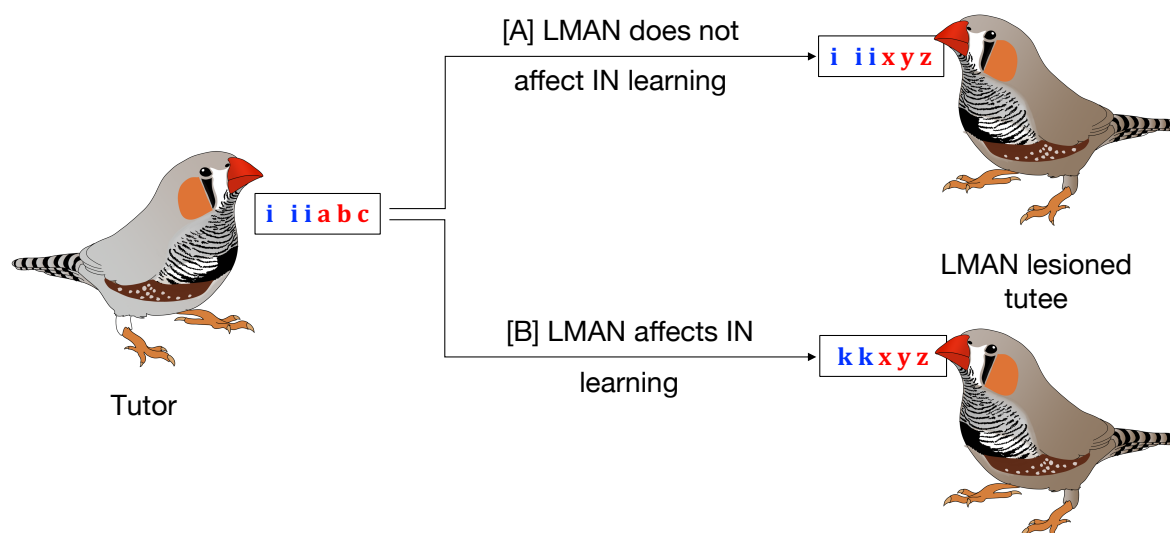


Figure 1.5: Schematic of expected outcomes of LMAN lesion.

‘i i i a b c’ represents the tutor’s song, where ‘i’ is the introductory note and ‘a b c’ are the motif syllables. If LMAN is not involved in the learning of INs, then the tutee will have INs similar to the tutor (represented as ‘i i i’ in [A]), but the tutee will not learn the motif syllables from the tutor (represented as ‘x y z’ in [A]). Conversely, if LMAN affects the learning of INs, then the LMAN lesioned tutee will not learn both INs and motif syllables from the tutor. In this case, both the INs and motif of the tutee will be different from the tutor (represented as ‘k k x y z’ in [B]).

The first objective of my thesis is to determine the neural coordinates and extent of the LMAN nucleus with respect to the custom-built stereotaxic apparatus that we have in our lab. This will increase the accuracy of hitting LMAN in every surgery, causing minimal damage to the nearby brain regions. My next objective is to perform bilateral electrolytic LMAN/sham lesions in

socially tutored juveniles during the song learning phase and quantify its effect on the final stereotyped song learned by the lesioned birds. Next, I want to standardize a staining method to visualize LMAN on histological sections. Finally, I want to confirm the lesions and quantify the percentage of LMAN left in the lesioned birds by analyzing the brain samples.

CHAPTER 2: Materials and Methods

Experimental procedures performed at IISER Pune were approved by the Institute Animal Ethical Committee in accordance with the guidelines of the Committee for Control and Supervision of Experiments on Animals (CCSEA, New Delhi, India).

2.1 Experimental birds

All adult zebra finches ($n = 19$) used for experiments were either bred in IISER Pune ($n = 11$) or bought from outside vendors ($n = 8$). All the juvenile zebra finches ($n = 34$ male juveniles from 10 different nests) used in the experiment were bred in IISER Pune. All the birds had a 14 hr day 10 hr night cycle with room temperature maintained at 25 °C and humidity levels between 60-80%. Antidromic stimulation was tried on four different birds ($n = 1$ adult and $n = 3$ juvenile zebra finches), bilateral electrolytic LMAN/sham lesion was performed on 33 birds ($n = 2$ adults and $n = 31$ juvenile zebra finches), and retrograde tracer injection was carried out on three birds ($n = 3$ adults).

2.2 Experimental design

Juvenile zebra finches begin to learn the song from their father around 25 days after hatching (Roper and Zann, 2006). Hence, to avoid any influence of the father's song on the juveniles, the father was separated from the nest when the juveniles were around 10 days old (range: post hatch day or phd 7-15, median: 11) days old ($n = 32$). All these hatchlings ($n = 32$) were further raised by their mother until post-hatch day 35 (range: 30-42, median: 35). After that, these juveniles were tagged and separated into individual cages and socially tutored by a predetermined adult male zebra finch with an extreme number of INs (<2 INs or >4 INs, Figure 2.1). As visual isolation (even with no acoustic isolation) can impede juveniles' song learning (Eales, 1989), all juveniles were visually separated from other birds in the colony except for their designated tutor during the song development phase (they could hear other birds). The father was not separated from the nest in a couple of exceptional cases. Juveniles in those nests were reared by both parents ($n = 2$) and were allowed to learn songs from their father.

All surgical procedures on juveniles (either bilateral LMAN/sham lesion or antidromic stimulation) were carried out after about a week of exposure to the tutor (range: 5-14, median: 7) or the father ($n = 32$ were exposed to tutor and $n = 2$ were with their father). Juveniles used for antidromic stimulation ($n = 3$) were older than 43 days post-hatch (range: 43-66, median:

47.5), and all juvenile finches used for bilateral lesions ($n = 21$ for LMAN lesion and $n = 10$ for sham lesion) were around 43 days old post-hatch (range: 38-48, median: 43). Songs were recorded from all these juveniles immediately before and after the lesion. The pre-surgery song was recorded starting a few days after exposing the juvenile to the tutor, and the surgical procedure was performed immediately the next day after getting sufficient song bouts. After the surgery, songs were recorded, and the juveniles were returned to their tutor with visual isolation from all the other birds in the colony. These juveniles were tutored further until post-hatch day 90 ($n = 22$), except for a few cases where the tutor died before the juvenile turned 90 days ($n = 2$). One tutor died when the juvenile was 39-41 days old, and another one died when the juvenile was 54-55 days old. After post-hatch days 90, undirected songs were recorded, and the birds were returned to the colony ($n = 20$ birds recorded, $n = 4$ yet to turn 90 days old). Undirected songs were further recorded after post-hatch day 120 ($n = 12$ birds recorded, $n = 12$ yet to turn 120 days old). After recording the post-hatch day 120 song, birds were euthanized ($n = 9$), and brains were sectioned and stained to visualize and quantify the LMAN nucleus. Undirected songs recorded from the tutored birds after post-hatch day 120 were used for all the analyses. A detailed schematic representation of this experimental design is shown in Figure 2.2.

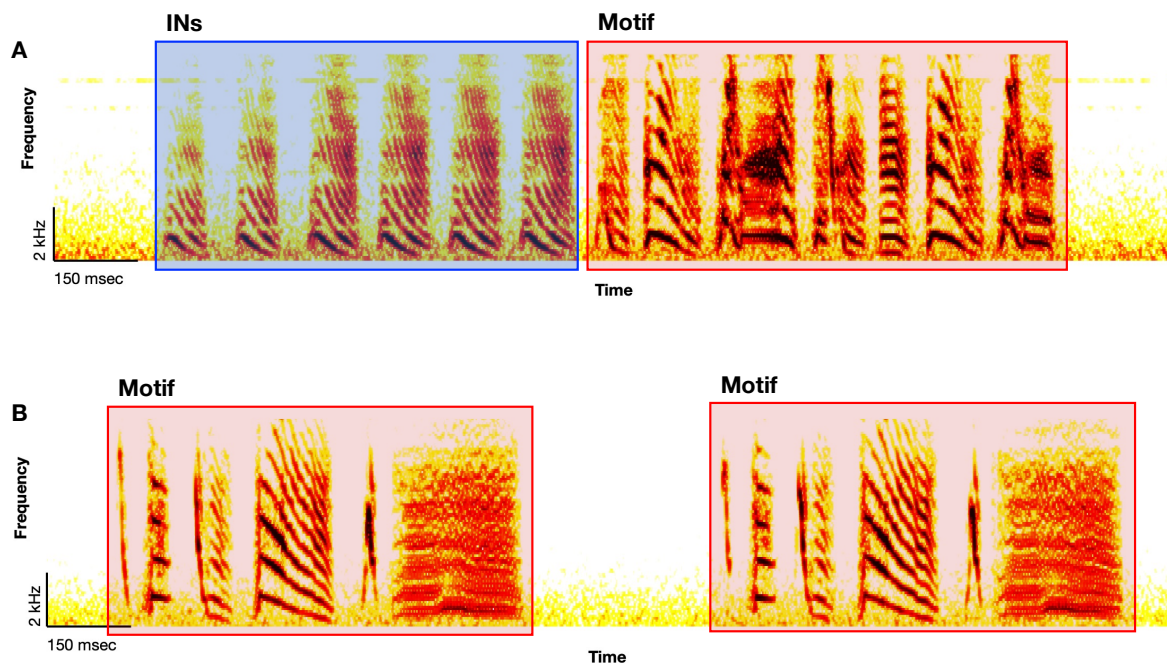


Figure 2.1: Spectrogram of adult male zebra finch song with extreme number of INs.

The INs are highlighted in blue, and the motifs are highlighted in red. A) song spectrogram of a high IN tutor where the bird produced 6 INs before the motif, and (B) shows the song spectrogram where the tutor produces a song without any IN (no IN bird). Scale bar: 150 msec (x-axis) and 2kHz (y-axis)

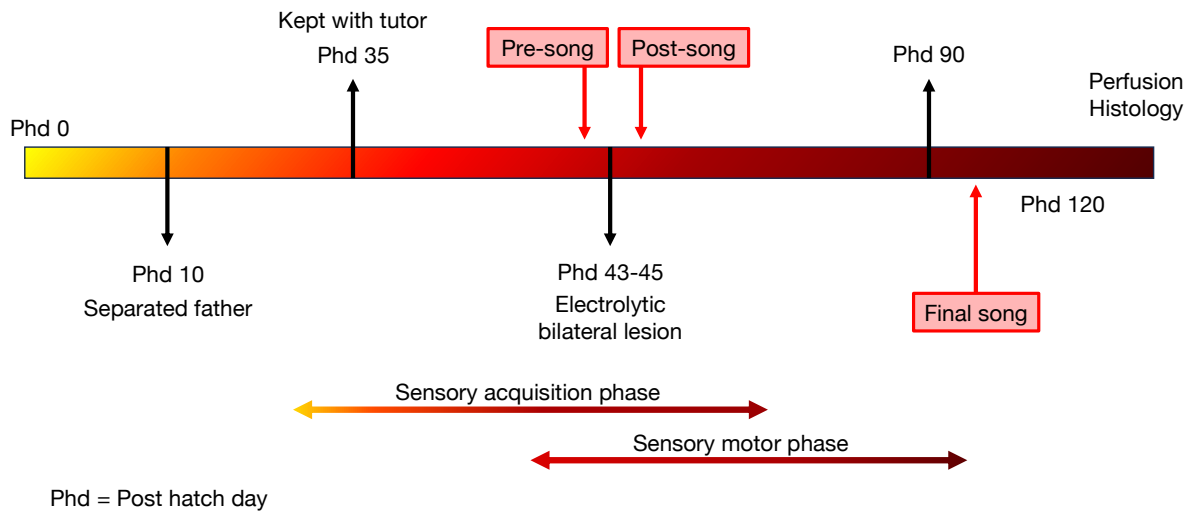


Figure 2.2: Schematic representation of the experimental design.

The father was separated from the nest when the juveniles were around 10 days old. The male juveniles were tagged and kept for tutoring with an adult male zebra finch when the juveniles were around 35 days post-hatch. The electrolytic bilateral lesion surgeries were performed when the juveniles were around 43-45 days old. Songs were recorded from the juveniles both immediately before and after the lesion. After that, the juveniles were kept further for tutoring until post-hatch day 90. The final song was recorded from these birds after post-hatch day 90 and after post-hatch day 120. Finally, these birds were transcardially perfused, and brains were sectioned, stained, and analyzed to quantify the lesions.

2.3 Tutors

Songs were recorded from adult birds (>90 days post-hatch) to find birds having songs with an extreme number of introductory notes (potential tutors). On average, juvenile zebra finches have an innate tendency to produce three INs irrespective of whether the bird was normally reared, socially tutored, or isolated (Kalra et al. in 2021). Hence, I actively looked for adult zebra finches with an extreme number of INs for their songs (either <2 or >4) to be used as tutors. This selection criteria was aimed at ensuring greater certainty in determining whether the juvenile birds learned the IN numbers from their tutors or if it was an inherent trait. For this, undirected songs were recorded from multiple birds in our colony, and 13 birds with an extreme number of INs were selected ($n = 6$ no IN birds, $n = 2$ low IN birds, $n = 5$ high IN birds).

2.4 Song Recordings

All songs were recorded by keeping a microphone (C417PP omnidirectional condenser microphone by AKG) directly above the bird's cage in a custom-made sound attenuation box

(NewTech Engineering Systems, Bangalore, India, or custom-built sound boxes from an ice cooler). A custom-written Python script was used to record songs from the birds at a sampling rate of 44,100 Hz. The audio signal recorded from the microphone was amplified using a mixer (Behringer XENYX 802) and subsequently digitized using a sound card. The audio was recorded in continuous mode (recorded in a loop as 30 seconds long .wav files) for an extended period to get a sufficient number of bouts. The digitized audio recordings were screened for songs, band-pass filtered, and labeled using custom-written MATLAB scripts. A minimum of 20 .wav files were labeled per bird, and bouts were picked (at least 20 bouts per bird) from these files for analysis. All the files used for analysis had >2 s of silence before and after the song bout.

For juvenile zebra finches, songs were recorded before ($n = 31$) and after ($n = 30$) the bilateral lesion surgery (both LMAN and sham lesion). The pre-lesion songs were recorded 2-3 days before the surgery when the juveniles were kept along with their tutors in an acoustic box. The post-lesion songs were recorded immediately after the lesion surgery for 2-3 days by isolating only the juvenile in the acoustic box. The final stereotyped song from these lesioned birds was recorded after post-hatch day 90 and after post-hatch day 120.

2.5 Surgical procedures

All surgical procedures were performed under aseptic conditions ($n = 5$ for antidromic stimulations, $n = 33$ electrolytic bilateral LMAN/sham lesion, $n = 3$ retrograde tracer injection). Birds were given analgesics (meloxicam, 0.25mg/Kg) orally at least an hour before the surgical procedure, and they were deprived of food during that period. Birds were anesthetized with inhalant isoflurane (3-4% for induction and 0.25-4% for maintenance) throughout the duration of surgery, and they were placed on a custom-built zebra finch stereotaxic apparatus (Narshige, custom-built) with their head restrained using ear bars and a beak bar. The body temperature of the birds was maintained throughout the procedure using a heat lamp. Feathers were plucked, and skin incisions and small craniotomies were performed above the mid-sagittal sinus and above the target nuclei after injecting local anesthesia (lidocaine, 25-50 μ L). After all the surgical procedures, the birds were monitored for successful recovery. Whenever the birds were found sick or weak following surgery, they were given appropriate care according to the veterinarian's instructions.

2.5.1 Antidromic stimulation

I used the antidromic stimulation technique (similar to those outlined by Hahnloser et al. in 2002) to find the coordinates of LMAN (Figure 2.3). Previous research suggests that there are neurons within LMAN that project to RA (Bottjer et al., 1989; Vates and Nottebohm, 1995). So, one would expect the current from electrical stimulations in RA to trace back to LMAN. My objective was to record this electrical activity within LMAN and, hence, obtain the coordinates for LMAN with respect to the custom stereotypic apparatus that we have in our lab.

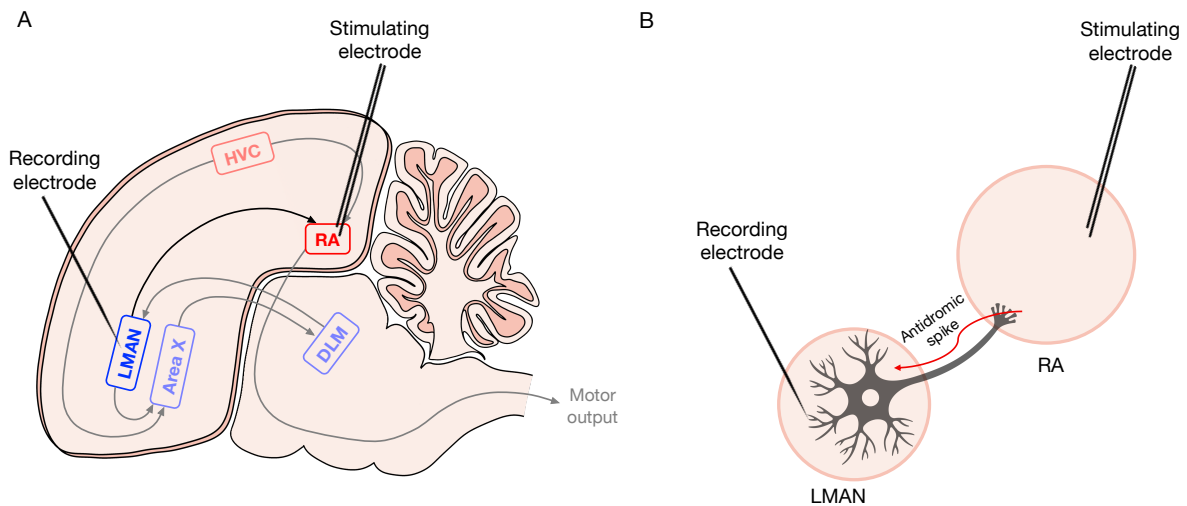


Figure 2.3: Schematic representation of antidromic stimulation in RA to locate LMAN.

A stimulating electrode (made of two tungsten microelectrodes with blunt tip) was inserted into RA for current stimulation. When RA is stimulated electrically, the current is expected to pass antidromically into LMAN due to neurons projecting from LMAN to RA (shown by the red arrow in B). A recording electrode (made with one tungsten microelectrode with a fine tip) was inserted into LMAN to record the antidromic spike.

Essentially, after anesthetizing and stabilizing the bird on the stereotaxic apparatus, small skin incisions and craniotomies were performed above the mid-sagittal sinus and above the coordinates of LMAN and RA, as indicated by the zebra finch stereotaxic atlas (Nixdorf-Bergweiler and Bischof, 2007). The location of RA was confirmed through anesthetized neural recording, identifying the characteristic continuous spiking activity of RA neurons. Subsequently, I inserted a stimulating electrode composed of two shanks (FHC tungsten microelectrodes with a blunt tip) glued together with a separation of $\sim 500 \mu\text{m}$ from each other

into RA. Next, I inserted a recording electrode (FHC tungsten microelectrodes with a fine tip) into LMAN and recorded the neural activity from multiple different coordinates within and around the expected LMAN nucleus region as per the zebra finch stereotaxic atlas (Nixdorf-Bergweiler and Bischof, 2007). A silver wire was placed under the skin as a reference or ground. After inserting the recording and the stimulating electrodes, I performed electrical stimulations in RA (200 μ A to 1 mA for a duration of 200 μ s) and recorded the electrical activity in LMAN. All electrical stimulations were done using a *flexible stimulus isolator (Iso-Flex by A.M.P.I.)* that was precisely controlled using *Arduino*. The recorded electrical signals were visualized on a PC using a data acquisition board from *Intan Technologies*. After the procedure, all the exposed regions were covered with sterile bone wax (Truwax S810) and further covered with acrylic repair material (DPI RR cold cure). A total of 5 antidromic stimulation surgeries (n = 2 adults and n = 3 juveniles) were attempted, of which the first two were performed on an adult bird (on either hemisphere), and the later ones were performed on juvenile birds. Table 2.1 provides detailed information on these surgeries.

Table 2.1: Details of the antidromic stimulation surgeries performed.

(All the coordinates for the lesion were marked with reference to the mid-sagittal sinus; LH = left hemisphere; RH = right hemisphere)

S. No.	Bird name	Coordinates tried for LMAN (mm)		Comments
		AP	ML	
1	red119white100 (LH)	4.8, 5, 5.2,	1.5, 1.8	Tried 4 different coordinates; no activity
2	red119white100 (RH)	4.6, 4.8, 5, 5.2	1.5, 1.8	Tried 7 different coordinates; no activity
3	green132 (LH)	4.4, 4.6, 4.8, 5, 5.2	1.5, 1.7, 1.9	Tried 8 different coordinates; got Area X like high-frequency firing patterns
4	green130 (RH)	4.2, 4.5, 4.7, 5	1.2, 1.5, 1.6, 1.7	Tried 6 different coordinates; got Area X like high-frequency firing patterns
5	green138 (LH)	4.675, 4.75, 4.875, 5, 5.2	1.5, 1.55, 1.7, 1.9	Tried 7 different coordinates; got a consistent change in the low-frequency component following RA stimulation in LH; got LMAN-like bursting firing patterns in RH

2.5.2 *Bilateral electrolytic lesions*

For permanent physical ablation of LMAN, I chose bilateral electrolytic lesions over chemical ablation since the former method gives more control over the lesion. The size of an electrolytic lesion is controlled by the amount of current and the duration for which one passes the current through the brain. In comparison, it is difficult to confine the spread of the neurotoxin to the target region in chemical ablation (Lomber, 1999). Sometimes, the neurotoxin spreads outside the target region, causing lesions outside the target region. Bilateral electrolytic lesion surgeries were performed on adult ($n = 2$) and juvenile ($n = 31$) zebra finches by stereotactically targeting either LMAN ($n = 2$ adults, $n = 21$ juveniles for LMAN lesion) or a location adjacent to LMAN ($n = 10$ juveniles for sham lesion). Each side had a total of 6 injection sites, with each site having lesions at one ($n = 28$) or two ($n = 5$) different depths. The lesion parameters used were 100 μ A for 50 sec ($n = 29$ juveniles, $n = 2$ adults), 60 sec ($n = 1$ juvenile), or 100 sec ($n = 1$ juvenile). Anesthetized neural recordings were performed for the initial surgeries at multiple coordinates and varying depths to delineate the LMAN nucleus ($n = 2$ juveniles). In these birds, the lesion coordinates were decided based on neural activity. Later, histological analyses were done to confirm lesion locations in these two birds. Tables 2.2, 2.3, and 2.4 provide details of all the lesion surgeries.

2.5.3 *Tracer injection*

Retrograde tracer injections were performed on adult zebra finches ($n = 3$ birds or $n = 6$ brain hemispheres) to fluorescently label LMAN (Figure 2.4). A 10,000 molecular weight retrograde tracer (Dextran Alexa Fluor 594) was injected into the RA nucleus using a programmable nanolitre injector (Drummond Nanoject III). Since LMAN projects to RA, we expect the retrograde dye to trace back and fluorescently label LMAN. The microinjection needles used for this had a tip diameter of around 30 microns, and they were fabricated from glass capillaries by a micropipette puller (P-97 by Sutter Instruments). Table 2.5 provides information on these surgeries.

Table 2.2: Details of the bilateral LMAN lesion surgeries performed on juvenile zebra finches
(All the coordinates for the lesion were marked with reference to the mid-sagittal sinus)

S. No.	Bird Name	Age (days post hatch)	Coordinates tried for LMAN (mm)			Current (μ A)	Duration (sec)
			AP	ML	Depth		
1	green127	45-48	4.6, 4.8, 5.0	1.6, 1.8	1.8, 1.9, 2.2, 2.3, 2.4	100	50
2	yellow112 (LH)	42-44	4.2, 4.4, 4.6	1.4, 1.5, 1.6, 1.8	1.8, 1.9, 2.0, 2.3, 2.4	100	100
3	yellow111	43-45	4.2, 4.4, 4.6	1.5, 1.8	1.9, 2.3	100	60
4	yellow113	45-47	4.2, 4.4, 4.6	1.5, 1.8	1.9, 2.3	100	50
5	red134	45-46	4.2, 4.4, 4.6	1.5, 1.8	1.9, 2.3	100	50
6	blue111	42-47	4.4, 4.65, 4.9	1.5, 1.8	2.3	100	50
7	red140	43-44	4.4, 4.65, 4.9	1.5, 1.8	2.3	100	50
8	blue115	41-43	4.4, 4.65, 4.9	1.5, 1.8	2.3	100	50
9	blue113	41-43	4.4, 4.65, 4.9	1.5, 1.8	2.3	100	50
10	pink164	42	4.4, 4.65, 4.9	1.5, 1.8	2.3	100	50
11	blue183	41-42	4.4, 4.65, 4.9	1.5, 1.8	2.3	100	50
12	orange110	42-43	4.4, 4.65, 4.9	1.5, 1.8	2.3	100	50
13	yellow141	40-43	4.4, 4.65, 4.9	1.5, 1.8	2.3	100	50
14	red179	40-43	4.4, 4.65, 4.9	1.5, 1.8	2.3	100	50
15	orange122	41-43	4.4, 4.65, 4.9	1.5, 1.8	2.3	100	50
16	blue126	41-44	4.4, 4.65, 4.9	1.5, 1.8	2.3	100	50
17	blue172	44-47	4.4, 4.65, 4.9	1.5, 1.8	2.3	100	50
18	blue173	45-48	4.4, 4.65, 4.9	1.5, 1.8	2.3	100	50
19	blue180	38-42	4.4, 4.65, 4.9	1.5, 1.8	2.3	100	50
20	orange130	40-42	4.4, 4.65, 4.9	1.5, 1.8	2.3	100	50
21	orange134	41-42	4.4, 4.65, 4.9	1.5, 1.8	2.3	100	50

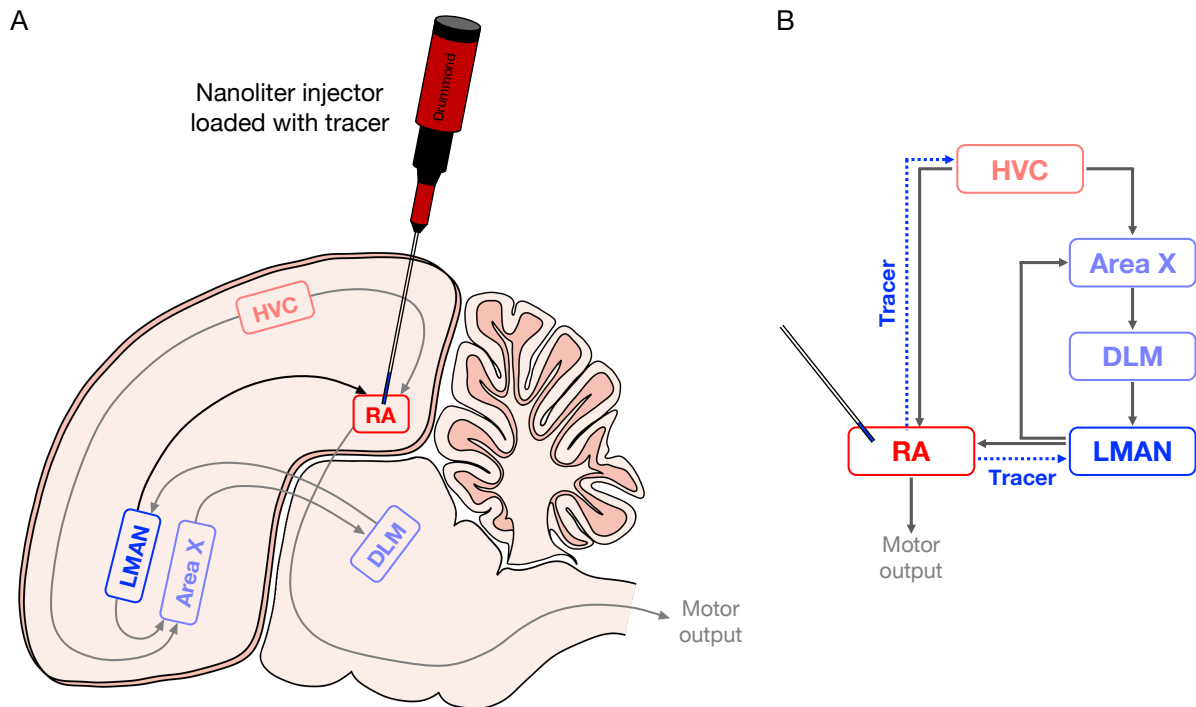
Table 2.3: Details of the bilateral LMAN lesion surgeries performed on adult zebra finches
(All the coordinates for the lesion were marked with reference to the mid-sagittal sinus)

S. No.	Bird Name	Age (days post hatch)	Coordinates tried for LMAN (mm)			Current (μ A)	Duration (sec)
			AP	ML	Depth		
1	green184white157	>90	4.4, 4.65, 4.9	1.6, 1.9	2.4	100	50
2	green183white156	>90	4.4, 4.65, 4.9	1.5, 1.8	2.3	100	50

Table 2.4: Details of the bilateral sham lesion surgeries performed on juvenile zebra finches

(All the coordinates for the lesion were marked with reference to the mid-sagittal sinus)

S. No.	Bird Name	Age (days post hatch)	Coordinates tried for sham (mm)			Current (μ A)	Duration (sec)
			AP	ML	Depth		
1	blue114	42-44	4.4, 4.65, 4.9	1.5, 1.8	1.3	100	50
2	orange109	41-42	4.4, 4.65, 4.9	1.5, 1.8	1.3	100	50
3	yellow143	41-44	4.4, 4.65, 4.9	1.5, 1.8	1.3	100	50
4	yellow144	41-44	4.4, 4.65, 4.9	1.5, 1.8	1.3	100	50
5	red190	41-44	5.1, 5.35, 5.6	2.0, 2.2	1.3	100	50
6	orange121	43-45	5.1, 5.35, 5.6	2.0, 2.3	1.3	100	50
7	orange129	42-44	5.1, 5.35, 5.6	2.0, 2.3	1.3	100	50
8	orange135	41-42	5.1, 5.35, 5.6	2.0, 2.3	1.3	100	50
9	blue175	40-43	5.1, 5.35, 5.6	2.0, 2.3	1.3	100	50
10	blue176	40-43	5.1, 5.35, 5.6	2.0, 2.3	1.3	100	50

**Figure 2.4: Schematic representation of retrograde injection into RA to fluorescently label LMAN.**

A fluorescent retrograde tracer was injected into RA using a programmed nanolitre injector. The tracer will travel (shown in B, blue dotted arrow) to LMAN (and HVC) due to neurons projecting from LMAN to RA (or HVC to RA), thus fluorescently labeling LMAN.

Table 2.5: Details of the retrograde tracer injection surgeries performed.

(LH = left hemisphere; RH = right hemisphere)

S. No.	Bird Name	Coordinates tried for LMAN (mm)			Total volume injected per hemisphere (nL)
		AP	ML	Depth	
1	green141white141	0, - 0.15 (LH) 0, - 0.2 (RH)	2.43	3.12	~ 300
2	green185white158	- 0.1 + 0.1	2.43	2.8 (LH) 2.6 (RH)	~ 300
3	yellow38	- 0.1 + 0.1	2.43	2.7	< 300

2.6 Perfusion and tissue preparation

Birds were deeply anesthetized with an overdose of isoflurane and transcardially perfused after the experiments with saline (0.9 % w/v) and further fixed with 4% paraformaldehyde in 1X PBS (Sigma P6148-500G). After two days of postfixing the brain in 4% paraformaldehyde, the brains were preserved in 30% sucrose in 1X PBS at 4°C. Some birds died a few days after the surgery. In such cases, the birds were directly decapitated, and then the samples were processed further, as mentioned above, for histology. Afterward, 40 µm thick sagittal sections of the brain were taken using a cryostat (Thermo Scientific CryoStar NX70 or Leica CM1950). The sections were sequentially collected in a 24-well plate and preserved in 1X PBS at 4°C.

2.7 Histology

All histology was done on sagittal sections (40 µm) of the brain stored in 1X PBS at 4°C, and every third section was used for staining. The brain sections were mounted on glass slides coated with 1:10 diluted poly-L-lysine (P8920-100ML, Sigma). Sections were first transferred into a Petri plate containing 1X PBS one by one and were mounted onto the slides using a paintbrush.

2.7.1 Immunohistochemistry

I tried Immunohistochemistry to stain for LMAN using an anti-calcitonin-gene-related-peptide antibody (CGRP by Sigma, C7113) as the primary antibody. Biotinylated secondary and HRP-

conjugated tertiary antibodies were used for further amplification (VectaStain ABC-HRP kit, PK-4002 by Vector Laboratories). Further, it was visualized using a DAB (3,3'-diaminobenzidine by Vector Laboratories, SK-4100) reaction. However this protocol is yet to be standardized.

2.7.1.1 Protocol 1

The sections were first taken in a 24-well plate and fixed with paraformaldehyde (4% PFA in 1X PBS) for 15 min. Next, the sections were washed thrice with phosphate buffer triton X-100 (0.3% PBT in 0.02M phosphate buffer) on a shaker for 10 min each for permeabilization. The sections were quenched for endogenous peroxidase by oxidizing the sections with hydrogen peroxide (0.5% H₂O₂ in PB) for 10 min in the dark. After this, the sections were washed with PBT (0.3%) for 5 min on a shaker, and blocking was done using normal goat serum (5% NGS in 0.3% PBT) for 1.5 hrs on a shaker. Following this, the sections were incubated in primary antibody (CGRP by Sigma, C7113) for 4 hrs at room temperature and then overnight or three nights at 4°C. Different primary antibody dilutions were tried in 2% NGS and 0.3% PBT. The next day, sections were given 3x PBT (0.3%) washes 10 min each on a shaker and then incubated in biotinylated secondary antibody (VectaStain ABC-HRP kit, PK-4002 by Vector Laboratories) for 45 min at room temperature. The secondary antibody solution was made by adding 150 µL of normal blocking serum stock and 50 µL of biotinylated secondary antibody to 0.3% PBT to make a 10 mL solution. After this, the sections were washed in PBT (0.3%) for 5 min on a shaker, and then the HRP-conjugated tertiary antibody was added. The tertiary antibody solution was made 30 min before use by adding 100 µL Reagent A (VectaStain ABC-HRP kit, PK-4002 by Vector Laboratories) and 100 µL Reagent B (VectaStain ABC-HRP kit, PK-4002 by Vector Laboratories) to 0.3% PBT to make 10 mL solution. The sections were incubated in the tertiary antibody for 30 min at room temperature. The sections were washed with PBT (0.3%) twice for 5 min each on a shaker to remove excess antibodies. Next, the 3,3' diaminobenzidine (DAB) reaction (DAB Peroxidase Substrate, SK-4100 by Vector Laboratories) was used for visualization. DAB was prepared freshly by adding 2 drops of DAB reagent 1, 4 drops of DAB reagent 2, 2 drops of DAB reagent 3, and 2 drops of DAB reagent 4 to 5 mL Milli-Q water. The DAB solution was added to the sections, and color was allowed to develop after which the reaction was stopped by washing with Milli-Q. All the sections were given 2x Milli-Q wash of 5 min each on a shaker followed by a 5 min PB (0.02M) wash on a shaker. The sections were stored in PB (0.02M) at 4°C and later mounted

on poly-L-lysine coated slides (Sigma, P8920-100ml) and air dried for 2-3 hrs. Next, the slides were immersed in 70 % ethanol for 1 min, 100 % ethanol for 2 min, and xylene for 1 min. Finally, coverslips were added after adding mounting media DPX and dried overnight. The next day, the excess DPX was removed, and the slides were cleaned with 70 % ethanol and imaged using a bright field microscope (Olympus MVX10).

This protocol worked once during standardization, and I got sufficient contrast to delineate LMAN and Area X from the background (Figure 2.5), but it did not work in later trials.

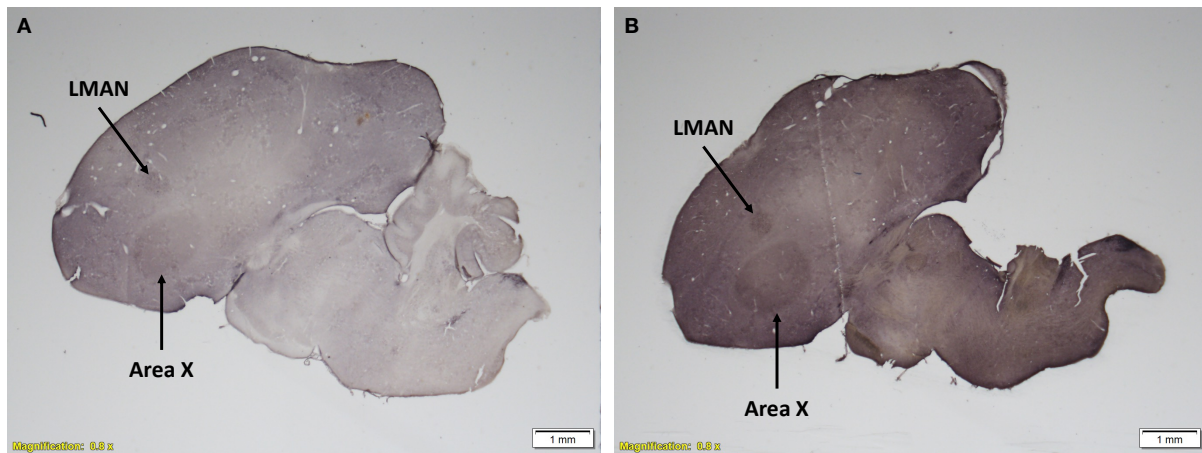


Figure 2.5: Immunohistochemistry (using protocol 1) of sagittal sections of zebra finch brain.

IHC done using CGRP as the primary antibody (1:5000) with (A) overnight incubation in the primary antibody and (B) 3 nights incubation in the primary antibody

2.7.1.2 Protocol 2

This protocol was provided by Mimi H. Kao (Tufts University, Medford, MA).

The sections of interest are taken in a 24-well plate and fixed with paraformaldehyde (4% PFA in 1X PBS) for 15 min, followed by three washes in PBT (0.3% PBT in 0.02M phosphate buffer) each for 10 minutes on a shaker. Next, blocking was done with normal goat serum (5% NGS in 0.3% PBT) for 1.5 hrs on a shaker. Then, the sections were incubated in primary antibody (CGRP by Sigma, C7113, or PV by Swant, PB235) for 4 hrs at room temperature and then overnight or three nights (only for CGRP) at 4°C. The next day, the sections were given PBT (0.3%) washes thrice each for 10 min on a shaker, followed by incubation in biotinylated secondary antibody (VectaStain ABC-HRP kit, PK-4002 by Vector Laboratories) for 1 hr at room temperature. The secondary antibody was used at a dilution 1:200 in 1.5% normal blocking serum stock and 0.3% PBT. After that, the sections were given 3x PBT (0.3%) washes each for 10 min on a shaker followed by 1hr incubation in HRP-conjugated tertiary antibody

at room temperature. The tertiary antibody solution was made 30 min before use by adding 100 μ L Reagent A (VectaStain ABC-HRP kit, PK-4002 by Vector Laboratories) and 100 μ L Reagent B (VectaStain ABC-HRP kit, PK-4002 by Vector Laboratories) to 0.3% PBT to make 10 mL solution. Next, the sections were washed thrice in PBT (0.3%) for 10 min each on a shaker. Next, the 3,3' diaminobenzidine (DAB) reaction (Sigma, D5637-1G) was used for visualization. The DAB reaction solution was prepared by adding 0.35 mL 0.4% NH_4Cl , 0.35 mL 40% β -D-glucose, 1.40 mL 1% NiAmSO_4 , 0.7 mL 25mg/mL DAB (Sigma, D5637-1G), and 34.20 mL 0.05 M TRIS to make the final volume of the solution to 37 mL. The sections were incubated in DAB reaction solution for 10 min, after which the DAB reaction solution was replaced by the glucose oxidase (GOD) reaction solution. The GOD reaction solution was made ~2 min before the 10 min incubation time in DAB got over, and it was prepared by adding 1.5 μ L GOD (10 K units, Sigma, G0543-10KU) to 30 mL DAB reaction solution. The GOD reaction was carefully monitored for any color development, and the reaction was stopped by washing with Milli-Q after getting sufficient contrast. The sections were given two quick rinses with Milli-Q followed by a 5 min wash in Milli-Q on a shaker. Then, the sections were washed with PB (0.02M) twice for 10 min each on a shaker and stored in PB (0.02M) at 4°C. These sections were later mounted on poly-L-lysine coated slides (Sigma, P8920-100ml) and air dried for 2-3 hrs. Next, the slides were immersed in 70 % ethanol for 1 min, 100 % ethanol for 2 min, and xylene for 1 min. Finally, coverslips were added after adding mounting media DPX and dried overnight. The next day, the excess DPX was removed, and the slides were cleaned with 70 % ethanol and imaged using a bright field microscope (Olympus MVX10).

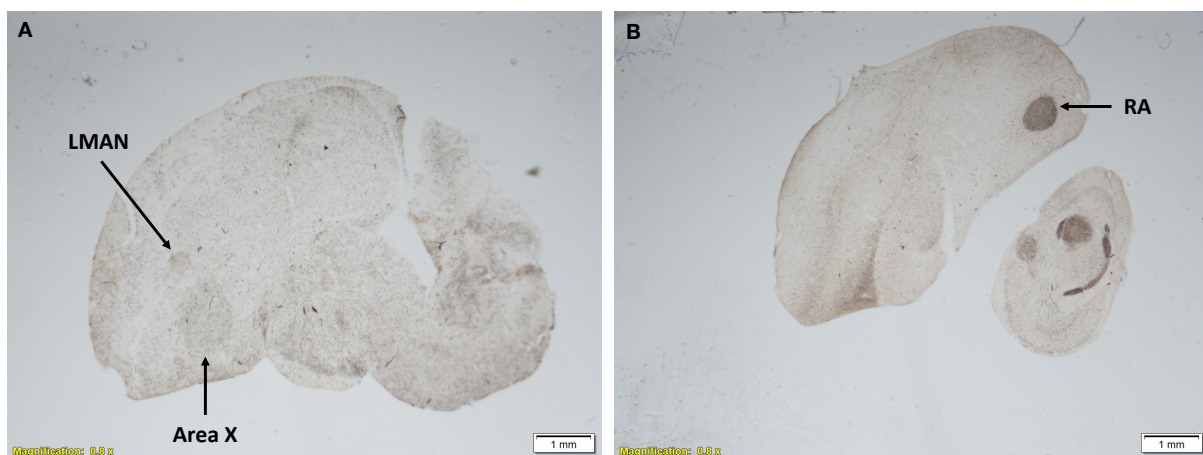


Figure 2.6: Immunohistochemistry (using protocol 2) of sagittal sections of zebra finch brain.
IHC done using (A) CGRP (1:10k) and (B) PV (1:10k) as primary antibody.

Using this protocol with CGRP as the primary antibody, LMAN (and Area X) can be visualized (Figure 2.6). However, some minor refinements are to be made to get better results. PV antibody can be used to visualize RA but not LMAN.

2.7.2 Cresyl violet staining

I performed cresyl violet acetate staining to visualize the LMAN nucleus on 40-micron-thick sagittal brain sections. The sections were first mounted on poly-L-lysine coated glass slides and dried overnight. A 1% cresyl violet acetate (Sigma, C5042-10G and SRL, 21442) solution was prepared by dissolving 1g cresyl violet acetate in 100 mL Milli-Q along with 0.539 ml of 100% acetic acid and 0.136g sodium acetate. This solution was kept on a stirrer overnight in the dark and filtered the following day using Whatman filter # 1 filter paper. The multiple different staining protocols tried are as follows:-

2.7.2.1 Protocol 1

The slides with the brain sections were first immersed in 1% cresyl violet solution for 10 min. Next, the slides were given 2-3 dips in Milli-Q water, followed by destaining for 1 min each in 50%, 50%, 70%, and 70% ethanol. Then, the sections were immersed in 95% ethanol for 1-1.5 minutes for further destaining. During this step, the slides were taken out at regular intervals and checked, and the destaining was stopped when I got a good contrast between known nuclei (like HVC, RA, Area X, or LMAN) from the background. After that, the slides were given one quick dip in 100 % ethanol and immersed in xylene for 15 sec. Finally, the sections were covered with coverslips after adding mounting media DPX and dried overnight. The next day, the excess DPX was removed, and the slides were cleaned with 70 % ethanol and imaged using a bright field microscope (Olympus MVX10).

Using this protocol, I could not reliably get a good contrast between LMAN and the background (Figure 2.7). Secondly, the duration of destaining in 95% ethanol could not be accurately defined. I always had to check the sections at regular intervals and decide on how long to destain in 95% ethanol, depending on the contrast.

2.7.2.2 Protocol 2

The slides with the sections were first immersed in xylene for 5 min, followed by 5 min in 100% ethanol, 2.5 min in 70% ethanol, 2.5 min in 20% ethanol, and 2 min in Milli-Q. Next,

the slides were dipped in 1% cresyl violet for 10 min. After that, the slides were given two dips in Milli-Q, two quick dips in differentiator I (270 mL 70% ethanol and 3 mL 100% acetic acid), one quick dip in differentiator II (270 mL 100% ethanol and 3 mL 100% acetic acid), one quick dip in 100% ethanol, and then the slides were kept in xylene for 15 sec. Finally, the mounting media DPX was added, and the sections were covered with coverslips and dried overnight. The next day, the excess DPX was removed, and the slides were cleaned with 70% ethanol and imaged using a bright field microscope (Olympus MVX10).

Using this protocol, I got a good contrast between LMAN and the background at the time of staining (Figure 2.8). However, in some cases, when the slides were left to dry overnight after adding a coverslip, the sections changed color (as shown in Figure 2.8 B), and the contrast between LMAN and the background was lost. Thus, this protocol also did not reliably stain for LMAN.

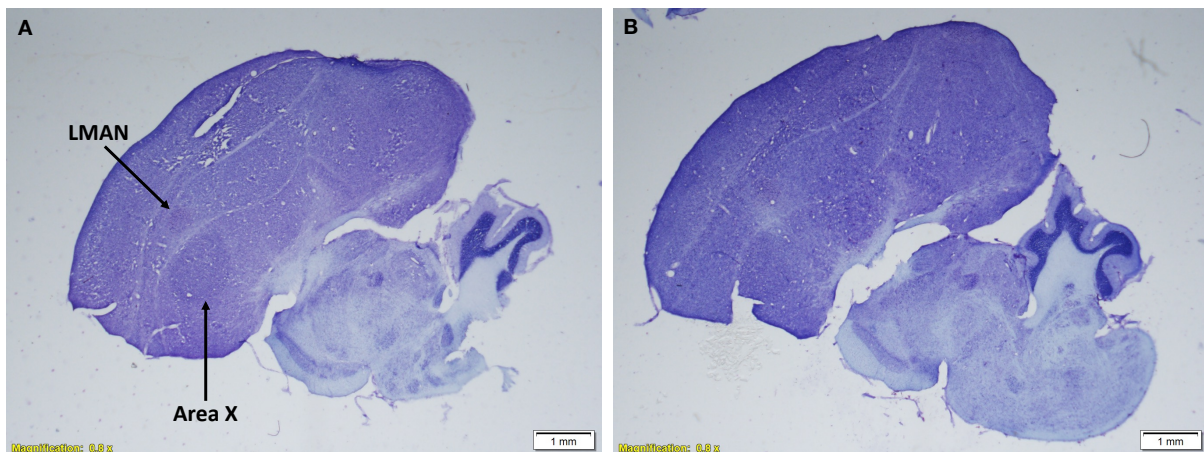


Figure 2.7: Cresyl violet stained sagittal sections of zebra finch brain using protocol 1.

A) shows the brain section where cresyl violet staining gave good contrast for LMAN and Area X from the background. The exact same protocol was followed for staining the sagittal sections from another bird, but I could not visualize LMAN (shown in B).

2.7.2.3 Protocol 3

This protocol was provided by Ashesh Dhawale (Centre for Neuroscience, IISc Bangalore). In this protocol, the cresyl violet solution (1%) used is heated to $\sim 60^{\circ}\text{C}$ using a water bath. The slides with brain sections are first dipped in xylene for 5 min, followed by 3 min each in 95% ethanol, 70% ethanol, and Milli-Q. Next, the slides are immersed in 1% cresyl violet solution (heated to $\sim 60^{\circ}\text{C}$) for 10 min. After that, the slides are kept in Milli-Q for 3 min, followed by destaining in 70% ethanol for 3 min and 95% ethanol for 2 min. After a quick dip in 100% ethanol, the slides were immersed in xylene for 2 min. Finally, the sections were covered with

coverslips after adding mounting media DPX and dried overnight. The next day, the excess DPX was removed, and the slides were cleaned with 70 % ethanol and imaged using a bright field microscope (Olympus MVX10).

This protocol gave better contrast for LMAN from the background (compared to the other two protocols), and it was altogether more reliable (Figure 2.9).

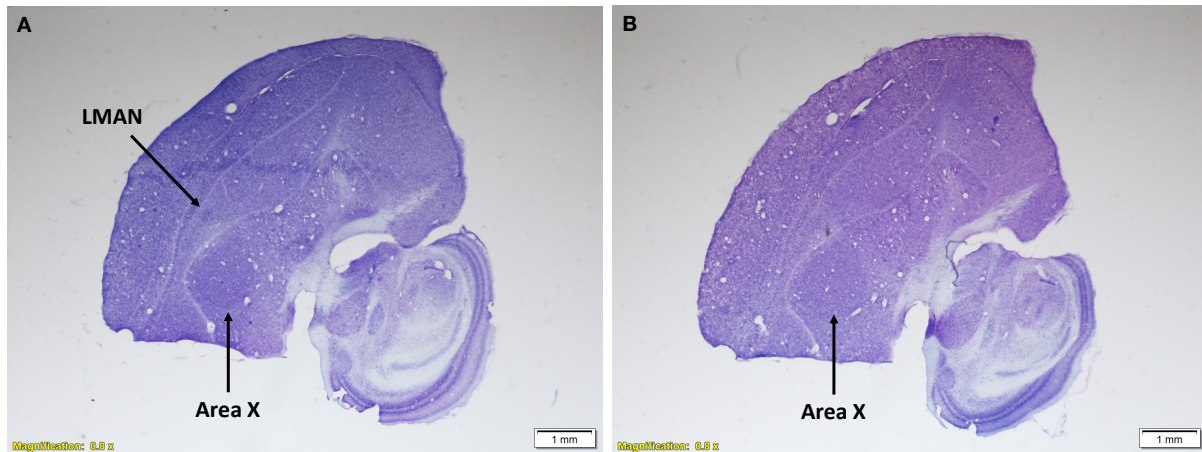


Figure 2.8: Cresyl violet stained sagittal sections of zebra finch brain using protocol 2.

LMAN and Area X can be distinguished from the background in (A), but LMAN cannot be distinguished from the background in (B).

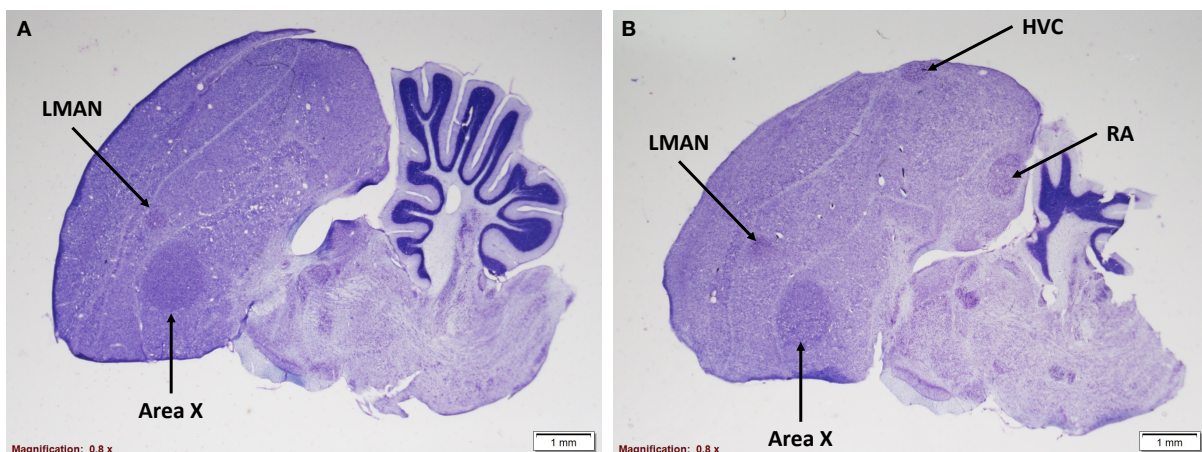


Figure 2.9: Cresyl violet stained sagittal sections of zebra finch brain using protocol 3.

A) shows LMAN and Area X, and (B) shows LMAN, Area X, HVC, and RA.

2.7.3 Quantification of LMAN remaining

I performed cresyl violet staining on 40 micron thick sections from LMAN/sham lesioned bird and then quantified the volume of LMAN remaining. The volume of LMAN remaining in each

hemisphere was normalized by the value obtained from a control brain sample stained with the same protocol. Histology was done for 8/24 birds ($n = 7$ LMAN lesioned and $n = 1$ sham lesioned), and the remaining birds are either yet to cross/crossed post hatch day 120 recently. Most of the birds with lesions in the LMAN ($n = 6/7$) have over 60% of their LMAN lesioned, while the bird with a sham lesion ($n = 1$) has all of its LMAN intact (Figure 3.8).

2.7.4 Tracer

All sample processing (sectioning and mounting) of the tracer injected birds was done in the dark. These brain sections ($40\ \mu\text{m}$ thick) were mounted onto poly-L-lysine coated glass slides. The excess PBS was soaked using soft tissue, and an antifade mounting medium (Vectashield, Vector Laboratories, H-1000-10) was used as it inhibits rapid photobleaching of fluorescent dyes. The coverslip was held in place using transparent nail polish. All these sections were stored at 4°C and imaged under a confocal microscope (Nikon Eclipse Ti) (Figure 2.10).

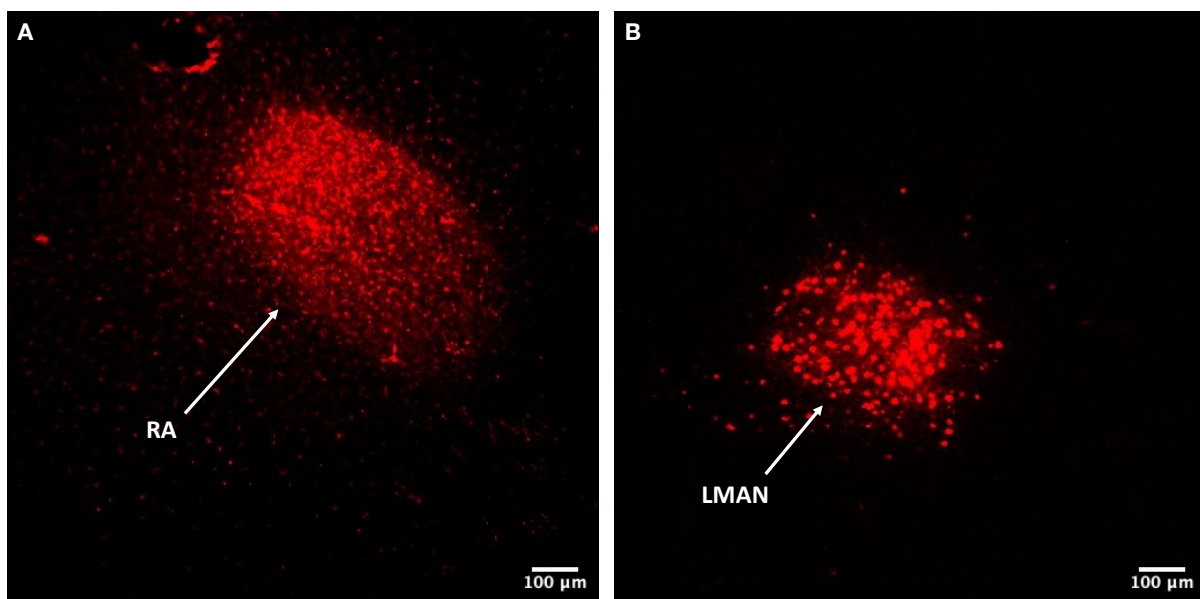


Figure 2.10: Confocal images from retrograde tracer injected bird.

A) shows RA (site of tracer injection), and (B) shows LMAN.

To summarise, IHC seems to be working on staining for LMAN, but the protocol needs to be standardized further for better contrast. Cresyl violet staining reliably stains for LMAN with the third protocol (see section 2.7.2.3). Although it is a quick and straightforward way to stain and check the fraction of LMAN remaining, this method does not give any idea about the amount of damage to projections going from LMAN to RA. This method is tricky, especially

for cases where the axons from LMAN to RA got damaged. Those birds will behave like LMAN lesioned birds, but cresyl violet stained sections will look similar to those of unlesioned birds. IHC or injecting a tracer will be more reliable in such situations. For the time being, I visualized the fraction of LMAN remaining using cresyl violet staining, but I will be performing IHC or retrograde tracer injection in the future.

2.8 Data analysis

All microscopy image analyses and LMAN quantifications were done using Fiji (Schindelin et al., 2012), an open-source image analysis platform. All song data analyses were done using custom-written MATLAB (Mathworks) scripts.

2.8.1 Song segmentation and syllable labeling

All the analyses were done using undirected songs, as these are songs produced by the birds in a more controlled condition without any external factors. All the songs were recorded in '.wav' format, and the birds were recorded for songs throughout the day in continuous mode. These recordings were first screened to select the files containing songs, and the selected files were segmented using an amplitude threshold. The script for automatic syllable segmentation considers any vocalization having a minimum duration of 10 ms and a minimum gap duration of 5 ms to be a syllable. Of these files with songs, 20 files were randomly selected for syllable labeling and further analyses. Syllables were automatically labeled using a template matching algorithm and checked manually for every vocalization. A bout of zebra finch song was defined by a sequence of vocalization that can be differentiated into INs and motifs and having a silence of 2 sec before and after the sequence of vocalization (as defined by Sossinka and Böhner, 1980). A typical bout may contain multiple motifs, and transition probability diagrams were constructed to identify the INs and motifs. For all analyses, the motif selected was the first motif of a bout, and the IN selected was the last IN before the first motif in a bout.

2.8.2 IN number calculation

Introductory notes were defined as the vocalization that repeats at the beginning of a song bout. Only the INs produced at the beginning of a bout before the first motif with ≤ 500 ms gap

between them were considered for calculating the mean IN number. The INs were counted starting with the last IN produced before the first motif in a bout and counted backward until either a vocalization different from the IN or an inter-syllable interval > 500 ms was encountered. The number of INs was calculated this way from 20 random bouts in the 20 labeled .wav files, and their average was calculated to get the mean IN number for a bird.

2.8.3 *IN acoustic structure*

All acoustic similarity measurements were done using a free open code software for the analysis of animal communication called Sound Analysis Pro 2011 (SAP2011, <http://soundanalysispro.com/>; Tchernichovski et al., 2000). INs and motifs were trimmed from the original .wav file, and acoustic similarity measurements were taken separately. The last IN produced before the first motif was used for IN similarity measurements by creating a new .wav file containing the selected IN with 10 ms silence before and after the vocalization. Ten such INs were randomly chosen from the labeled files for IN acoustic similarity measurement. The first motif in a song bout was used for motif similarity measurements by creating a new .wav file containing this selected motif with 10 ms silence before and after it. Ten such motifs were chosen randomly for motif acoustic similarity measurement from the labeled files. In some cases where the INs and motifs cannot be identified, the first 1.5 sec of the song bout was trimmed with 10 ms of silence before and after the sequence of vocalization for similarity measurements. In SAP, asymmetric time course similarity measurements were done for motifs as this accommodates potential differences in syllable order between the tutor and tutee. For instance, if the tutor's song had three syllables in a particular order and the juvenile learned all three syllables in a different order, the software would still give a similarity score of 100%. At the same time, if a pupil learned only a fraction of the tutor's song, this similarity measurement will give a fractional score corresponding to the percentage of vocalizations learned. For INs, symmetric time course similarity measurements were done using SAP. In cases where a bird produced two different types of INs, similarity measurements (symmetric time course) were done separately for the two types of INs and the total percentage similarity was taken as the average of the two similarity values.

CHAPTER 3: Results

This is collaborative project with Vidushi Sharma and Shikha Kalra

3.1 Standardizing LMAN coordinates

3.1.1 Location of LMAN was identified using antidromic stimulation and tracer injection in RA

To get the accurate coordinates for the LMAN with respect to our custom-built stereotaxis apparatus, I tried antidromic stimulation in RA while simultaneously recording from LMAN ($n = 2$ in adults, $n = 3$ juveniles, see Table 1 in Methods). As mentioned earlier, LMAN projects to RA, hence stimulation in RA would generate an antidromic spike in LMAN. RA was identified by characteristic spontaneous firing activity (Figure 3.1), and the stimulating electrode was inserted to this location. In 4/5 surgeries, I did not find any antidromic spike following stimulation in RA. However, I got high-frequency firing pattern, which were similar to the firing patterns of Area X in zebra finches (anesthetized extracellular neural activity). In the fifth surgery, I tried seven different coordinates in the left hemisphere of the brain for LMAN. I got a consistent change in voltage following stimulations in RA. Overall, the final coordinates used were AP: 4.7 mm, ML: 1.55 mm, and depth: 2.4 mm.

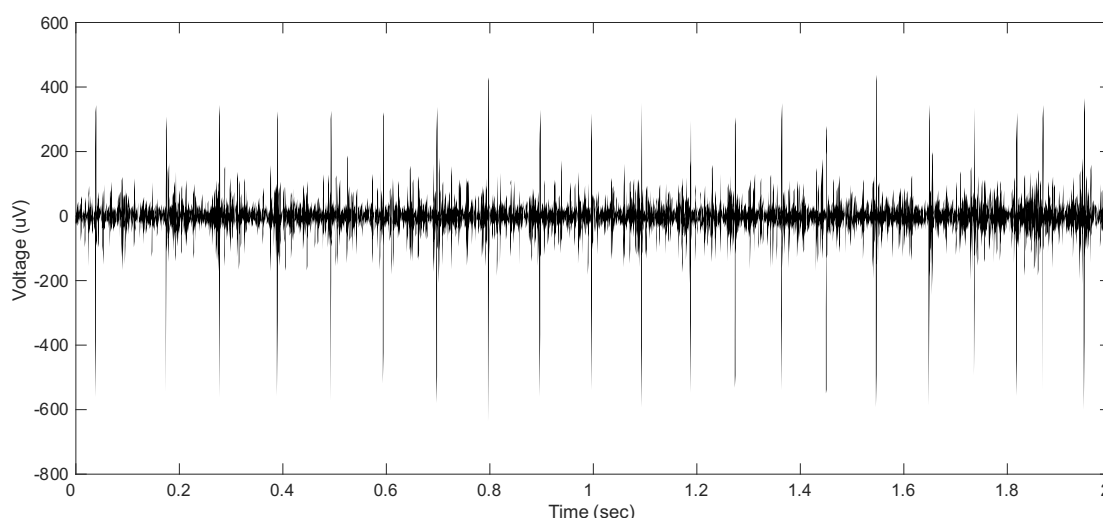


Figure 3.1: Spontaneous firing activity recorded from RA

Sample trace recorded from RA using a tungsten electrode during one of the antidromic stimulation surgeries to confirm the location of RA. X axis: time in seconds and Y axis: potential difference in microvolts

Further, I recorded extracellular neural activity in the same coordinate from the right hemisphere of the same bird under anesthesia. Here, I got LMAN-like bursting firing patterns for the coordinates AP: 4.7 mm, ML: 1.55 mm, and depth: 2.3 mm. Later, the location of the stimulating electrode was confirmed to be in RA, and that of the recording electrode was confirmed to be in LMAN on cresyl violet stained brain sections from this bird (Figure 3.2).

Following this, I also confirmed the location of LMAN by performing fluorescent retrograde tracer injections in RA (n = 6 brain hemispheres) (Figure 2.10).

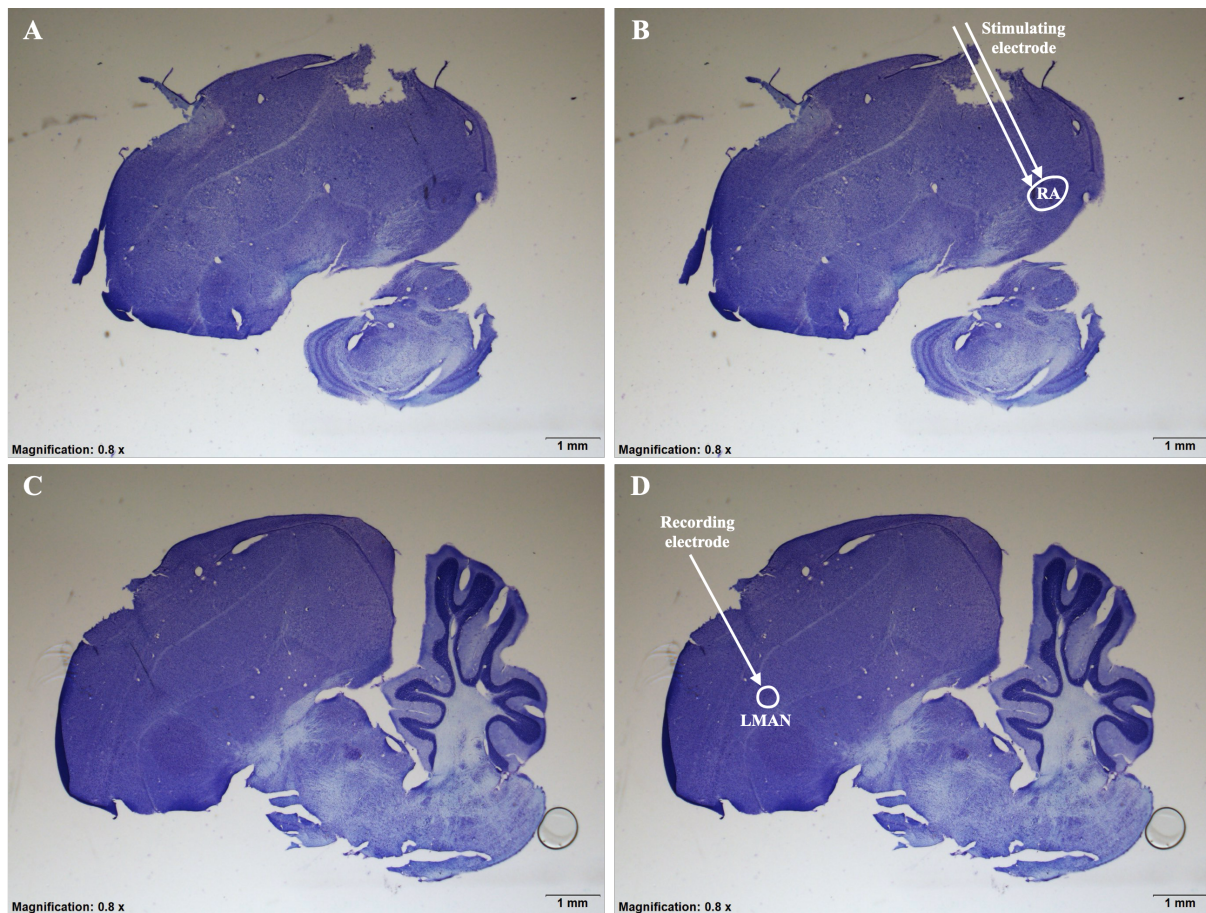


Figure 3.2: Sagittal brain sections (40 μ m thick) of zebra finch brain stained with cresyl violet acetate showing locations of stimulating electrode and recording electrode.

A) shows intact RA and the two stimulating electrode tracks pointing at RA, and (B) shows the same figure with the location of RA and the stimulating electrode highlighted. C) shows intact LMAN and the recording electrode tracks pointing at LMAN, and (D) shows the same figure with the location of LMAN and the recording electrode highlighted.

3.1.2 Standardization of lesion parameters

In order to standardize the lesion parameters, initially, I made multiple lesions (100 μ A for 50 sec each) around the LMAN coordinates. I made twelve lesions in each hemisphere of the brain (two lesions at two different depths for six different coordinates) to ensure that LMAN is completely lesioned bilaterally. The animal was perfused, and brain sections were stained with cresyl violet acetate to quantify the size and location of the lesion. The diameter of the LMAN in a control bird was found to be ~400 microns, but the diameter of the lesion was >1200 microns (Figure 3.3).

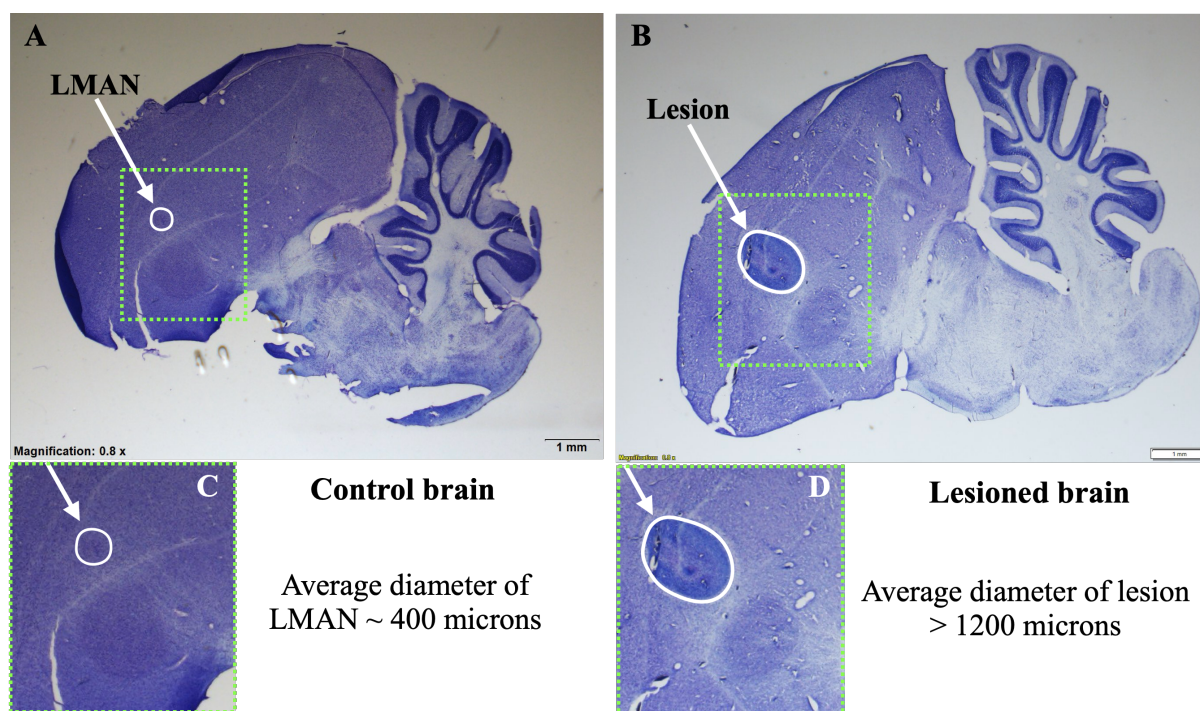


Figure 3.3: Sagittal brain sections (40 μ m thick) of zebra finch brain stained with cresyl violet acetate comparing the size of LMAN and lesion.

A) shows the location and size of intact LMAN in a control bird, and (C) is a zoomed-in view of the region marked by the green dotted line in (A). B) shows the location and size of the lesion, and (D) is a zoomed-in view of the region marked by the green dotted line in (B).

Since these lesions were huge and covered a large area, the LMAN nucleus was not visible in the lesioned brain sections. Hence, these sections were superimposed with corresponding cresyl violet stained sagittal brain sections from a control bird to get the approximate location of LMAN (Figure 3.4). These images show that the lesions were relatively big compared to the size of LMAN and damaged a lot of regions above and posterior to LMAN (Figure 3.4). Based on all these calculations and approximations, certain refinements were made to LMAN coordinates (see Table 3.1) and the amount of lesion. With the new coordinates, there are lesions at six locations in one hemisphere of the brain, with each coordinate having a lesion at a single depth. Here, the size of the lesion is much smaller and covers LMAN (Figure 3.5).

Table 3.1: LMAN coordinates

	AP	ML	Depth
Old coordinates	4.6, 4.8, 5.0	1.6, 1.8	1.8, 2.3
New coordinates	4.4, 4.65, 4.9	1.5, 1.8	2.3

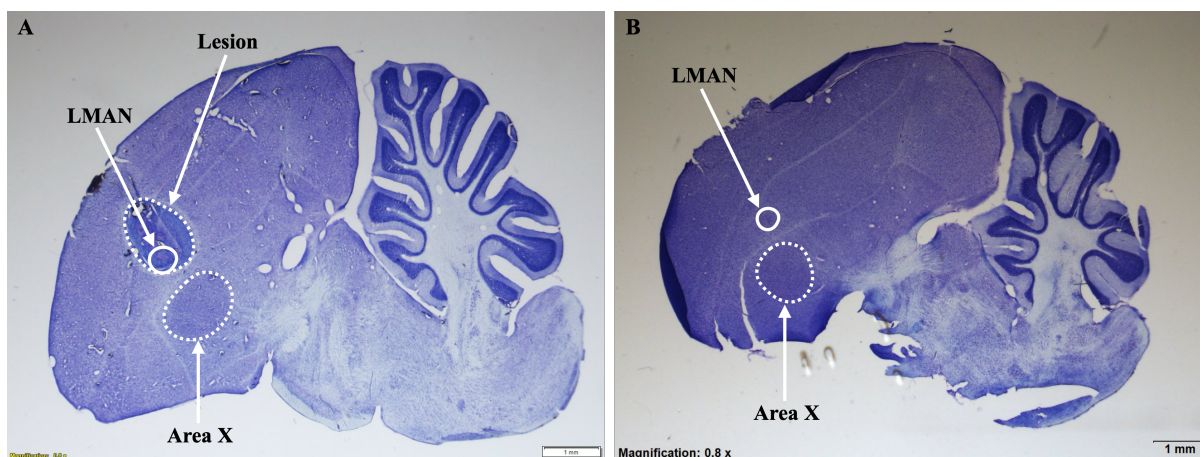


Figure 3.4: Sagittal brain sections (40 μ m thick) of zebra finch brain stained with cresyl violet acetate showing LMAN and lesion.

A) shows the location and size of the lesion with respect to the approximate location of LMAN and Area X. The location of LMAN in (A) was approximated by superimposing the cresyl violet stained sagittal brain section from a control bird (shown in B).

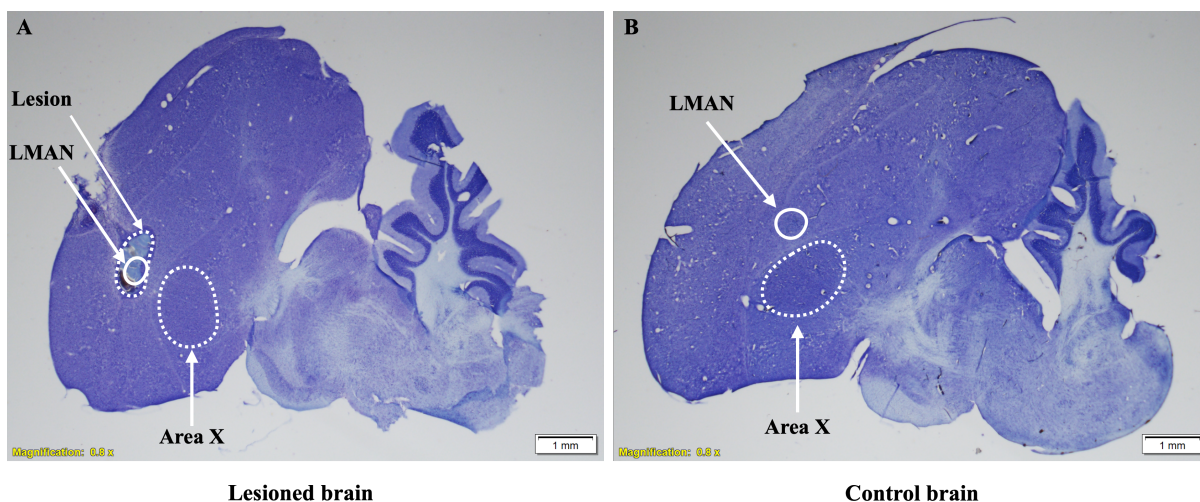


Figure 3.5: Sagittal brain sections (40 μ m thick) of zebra finch brain stained with cresyl violet acetate showing LMAN and lesion with the new coordinates.

A) shows the location and size of the lesion relative to LMAN with the new LMAN coordinates, and (B) shows the location and size of intact LMAN in a control bird that was used to approximate the location of LMAN in (A).

3.2 Visualized and quantified LMAN using cresyl violet staining

3.2.1 LMAN is observable in birds with sham lesions but not in birds with LMAN lesions following cresyl violet staining

I wanted to visualize and quantify the fraction of LMAN remaining in each of the lesioned birds. For this, I tried various protocols for both IHC and cresyl violet staining. Ultimately, the third cresyl violet staining protocol, provided by Ashesh Dhawale, worked reliably for me (see

sections 2.7.1 and 2.7.2 in Methods). Hence, I used this protocol to stain and visualize LMAN in lesioned birds. In LMAN lesioned birds, cresyl violet stained sections either did not show up LMAN in any of the sagittal sections (Figure 3.6) or LMAN was visible only in a few sections, confirming that a major fraction of LMAN got lesioned. Conversely, in sham lesioned birds, LMAN can be clearly visualized in multiple sections (Figure 3.7), confirming that LMAN was intact in those birds.

Histology was done for 8/24 birds ($n = 7$ LMAN lesioned and $n = 1$ sham lesioned), and the volume of LMAN remaining was quantified in each hemisphere using Fiji (Schindelin et al., 2012). The majority of the LMAN lesioned birds ($n = 6/7$) have more than 60% of their LMAN lesioned, whereas the sham lesioned bird ($n = 1$) has all of its LMAN intact (Figure 3.8).

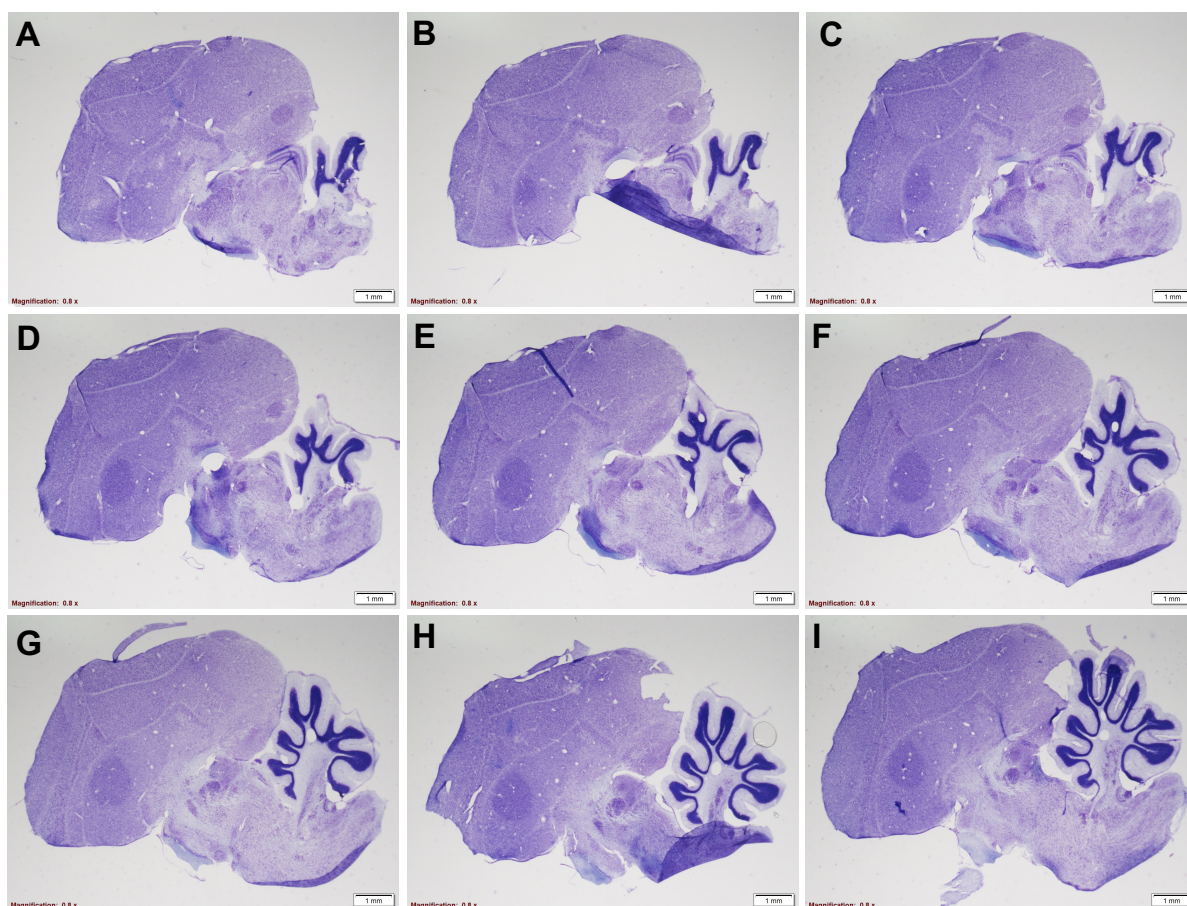


Figure 3.6: Sagittal brain sections (40 μ m thick) of LMAN lesioned zebra finch brain stained with cresyl violet acetate

A) to (I) are sequential images of sagittal brain sections from one hemisphere of an LMAN lesioned bird stained with cresyl violet acetate. Each section is 40 μ m thick, and every third section was stained.

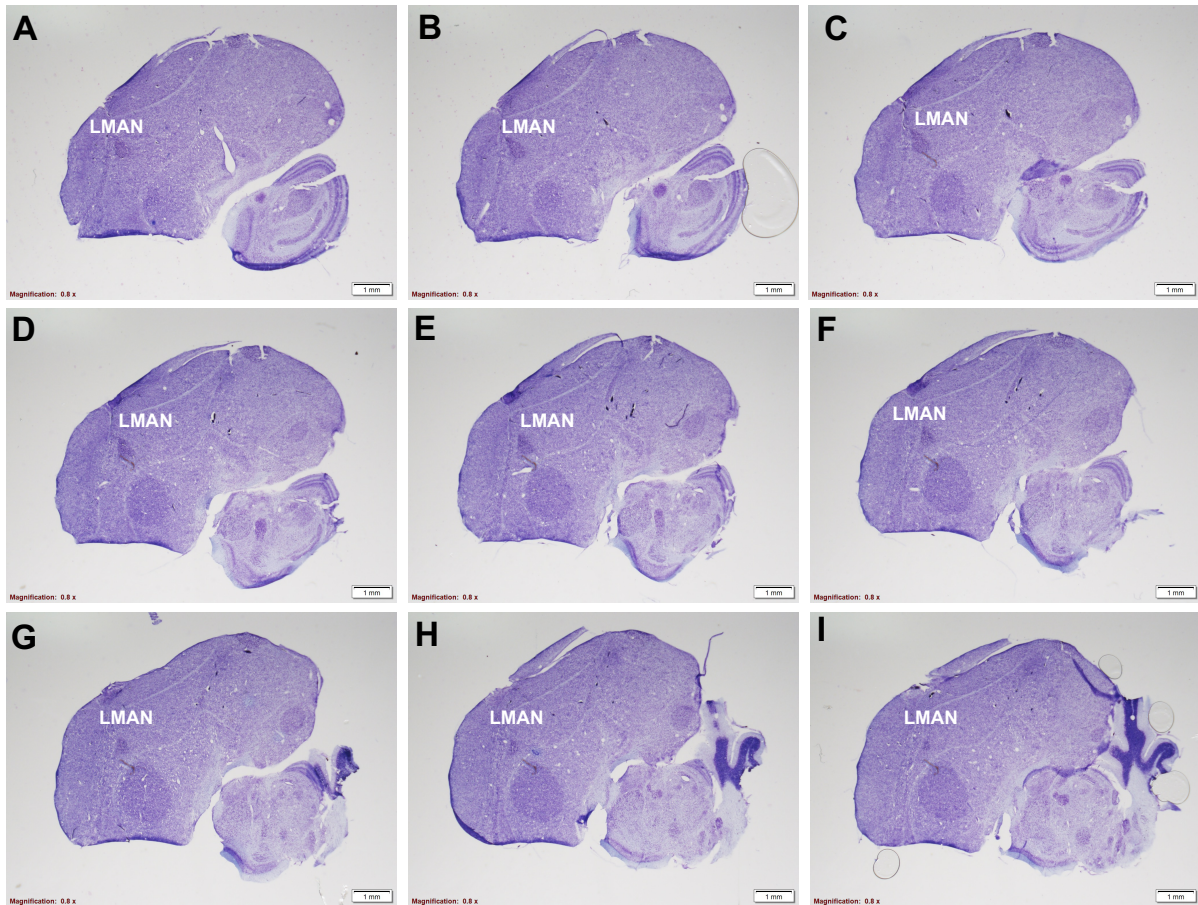


Figure 3.7: Sagittal brain sections (40 μ m thick) of sham lesioned zebra finch brain stained with cresyl violet acetate

A) to I) are sequential images of sagittal brain sections from one hemisphere of a sham lesioned bird stained with cresyl violet acetate. Each section is 40 μ m thick, and every third section was stained.

3.3 Lesions of LMAN affects learning acoustic structure of INs and motifs

To check if lesions of LMAN during the song learning phase affect learning the acoustic structure of motifs and INs, I compared the songs recorded from the lesioned pupils after post hatch day 120 with the song of their tutor.

3.3.1 Song spectrogram of sham lesioned pupil versus its tutor

The spectrogram of the sham lesioned bird looked qualitatively similar to the song spectrogram of its tutor (Figure 3.9). The IN in the pupil's song (Figure 3.9, highlighted in blue) looks qualitatively similar to that of the tutor. The pupil's song has only three syllables, of which the

first two (Figure 3.9, highlighted in red and green) look qualitatively similar to the first two syllables in the tutor's song. This suggests that the pupil may have learned song from its tutor.

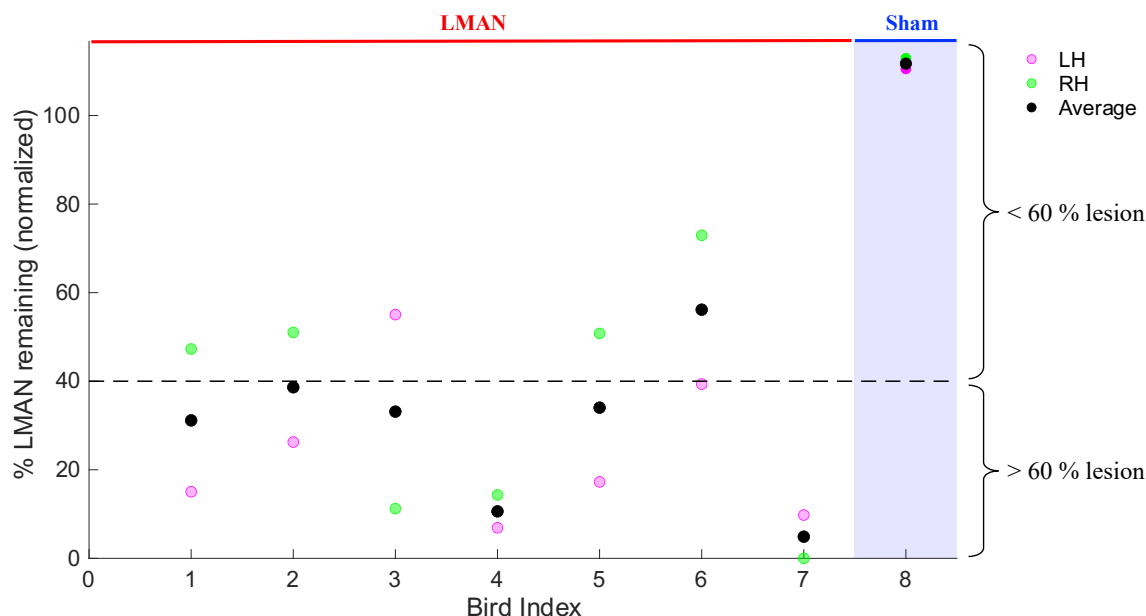


Figure 3.8: Volume of LMAN remaining

This graph shows the normalized volume of LMAN remaining in the left hemisphere (magenta circles), right hemisphere (green circles), and averaged across both hemispheres (black circles) for each bird. Bird index numbers 1-7 are LMAN lesioned birds, and the eighth bird (highlighted in blue) is a sham lesioned bird. The dotted line corresponds to 40% LMAN remaining (or 60% lesioned).

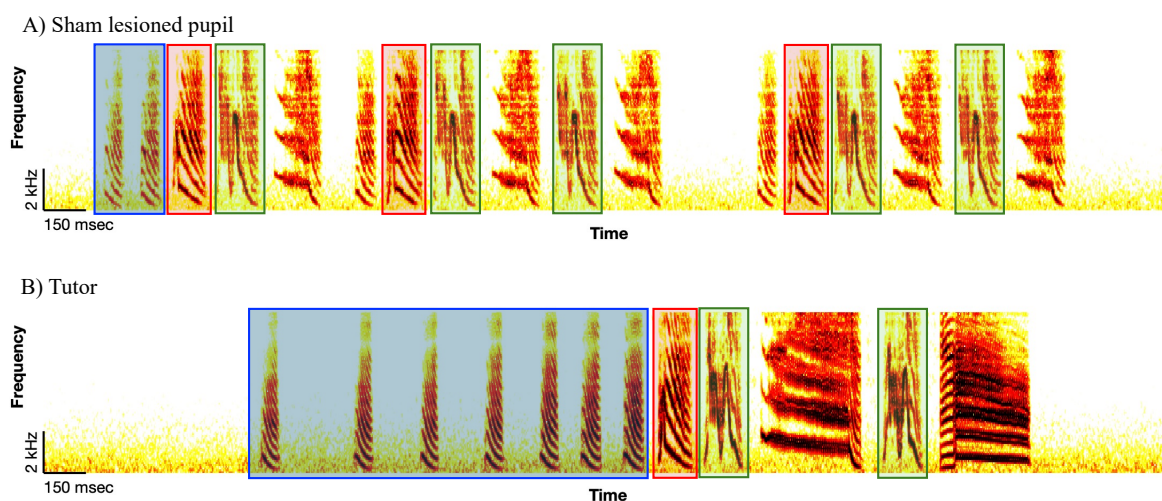


Figure 3.9: Song spectrogram of a sham lesioned pupil and its tutor

A) shows the song spectrogram of the sham lesioned pupil, and (B) shows the song spectrogram of the tutor with whom the pupil was tutored during the song learning phase. The IN (highlighted in blue) and a few syllables (highlighted in red and green) in the tutor's song look qualitatively similar to the IN and syllables in the pupil's song. Scale bar in (A) and (B): 150 msec (x-axis) and 2kHz (y-axis)

3.3.2 Song spectrogram of LMAN lesioned pupil versus its tutor

The song spectrogram of LMAN lesioned pupil looks qualitatively different compared to the song spectrogram of its tutor (Figure 3.10). The pupil has fewer number of syllables repeating multiple times for its song, whereas the motif in the tutor's song can be differentiated into multiple different syllables.

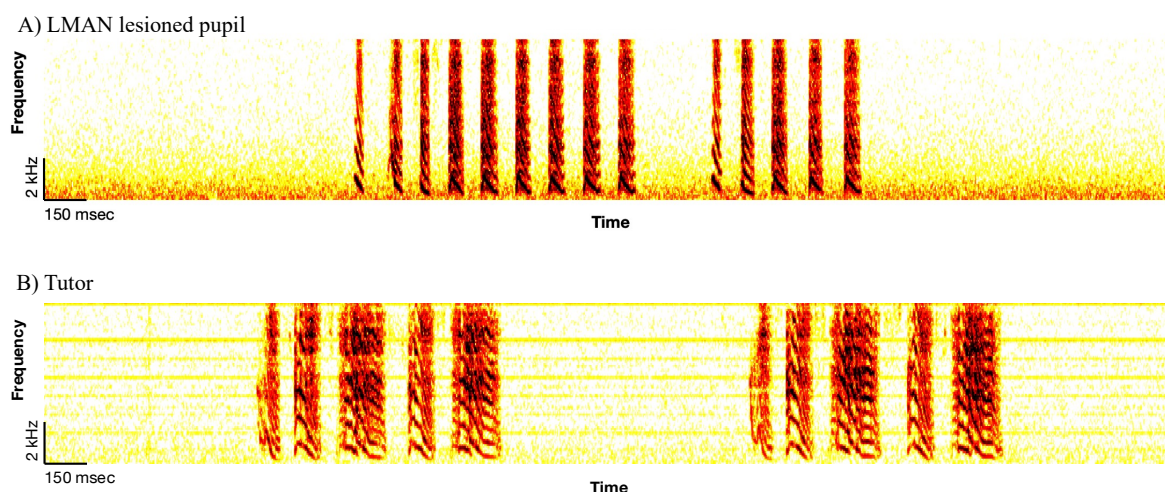


Figure 3.10: Song spectrogram of LMAN lesioned pupil and its tutor

A) shows the song spectrogram of the LMAN lesioned pupil, and (B) shows the song spectrogram of the tutor that the pupil was tutored with during the song learning phase. Scale bar in (A) and (B): 150 msec (x-axis) and 2kHz (y-axis)

3.3.3 Motif similarity is much lower for LMAN lesioned birds versus socially tutored birds

I wanted to check if lesioning LMAN during the song learning phase affects learning the acoustic structure of motif syllables, similar to what other people have shown previously. For this, I recorded the final song from the pupil after post hatch day 120 and compared the acoustic similarity of motif with that of its tutor using a software called *Sound Analysis Pro* (asymmetric, time course similarity measurements using SAP2011, <http://soundanalysispro.com/>; Tchernichovski et al., 2000). The lesioned birds were divided into two groups – those with LMAN lesions and those with sham lesions – depending on the coordinates used for lesioning. For comparison, I analyzed motif similarity data from socially tutored birds that were tutored by a social tutor without any surgical intervention. The motif similarity of LMAN lesioned birds ($n = 14$) was found to be significantly lower compared to the socially tutored birds ($n = 10$), but not the sham lesioned birds ($n = 7$) (Kruskal-Wallis, $p =$

0.0009; Post Hoc Tukey-Kramer) (Figure 3.11). Overall, this suggests that LMAN is essential for learning the acoustic structure of motif syllables from a tutor by the pupil. This outcome aligns with findings previously demonstrated by others in the field (Scharff and Nottebohm, 1991; Bottjer et al., 1984).

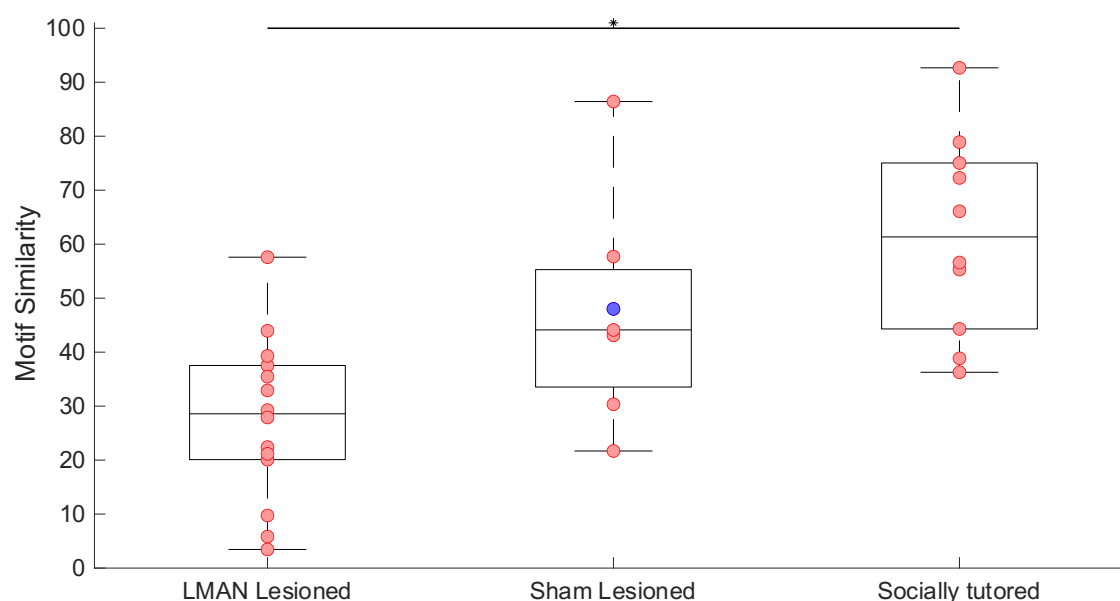


Figure 3.11: Motif similarity of LMAN/sham lesioned and socially tutored birds.

The lesioned birds are divided into LMAN/sham lesioned depending on the coordinates used for the electrolytic lesion. Each point on the graph represents the average motif similarity for a given bird with its tutor ($n = 14$ LMAN lesioned, $n = 7$ sham lesioned, and $n = 10$ socially tutored). There was an exceptional case in the sham lesioned category where the tutor died early in the song learning phase of the pupil, and the motif similarity of the same has been highlighted in blue. The motif similarity of LMAN lesioned birds is lower compared to socially tutored birds (Kruskal-Wallis, $p = 0.0009$). Post Hoc Tukey-Kramer, $p = 0.0960$ (Group 1-2), $p = 0.0007$ (group 1-3), $p = 0.4885$ (group 2-3). Socially tutored birds' data: Shikha Kalra

3.3.4 Unilateral lesions of LMAN may also affect learning the acoustic structure of motif syllables

Since LMAN is essential for learning the acoustic structure of motif syllables, I was curious to know if the pupil can learn the acoustic structure of motif syllables even if some fraction of LMAN is remaining in either hemisphere of the brain. For this, I independently quantified the volume of LMAN remaining in either hemisphere of the brain. The percentage of LMAN remaining was calculated by normalizing the volume of LMAN in lesioned birds with the volume of LMAN computed using the same procedure on a control bird with an intact LMAN.

When a significant fraction of LMAN is lesioned, the motif similarity is also very low (Figure 3.12). Even in the case where 55% of LMAN is remaining in the left hemisphere, but only ~11% of LMAN is remaining in the right hemisphere, the average motif similarity is very low (9.74%). Thus, if a significant fraction of LMAN is lesioned in either hemisphere, the bird may not be able to learn the song from its tutor. However, I need a larger sample size to draw any firm conclusions.

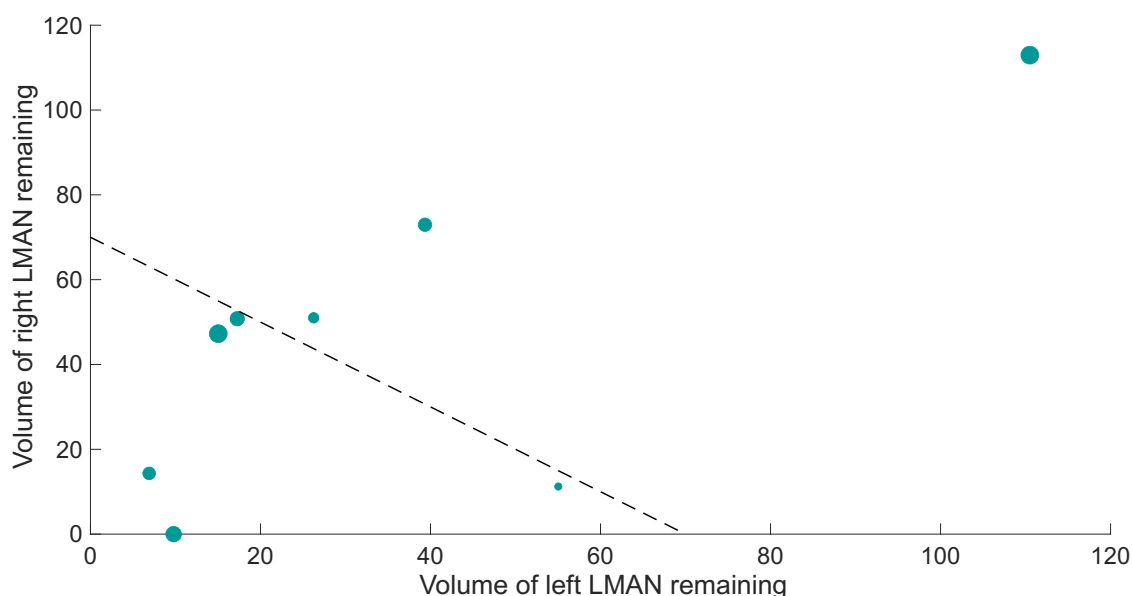


Figure 3.12: Fraction of LMAN remaining in the left hemisphere versus the right hemisphere compared to the motif similarity between the tutor and the tutee.

The x-axis shows the percentage volume of LMAN remaining in the left hemisphere, and the y-axis shows the percentage volume of LMAN remaining in the right hemisphere. Each point on the graph represents an individual bird, and the size of each point corresponds to the average percentage motif similarity between that given bird and its tutor. The dotted line corresponds to 35% of LMAN remaining averaged across both hemispheres.

3.3.5 *IN similarity is much lower for LMAN lesioned bird versus socially tutored bird*

A recent study suggested that the pupil can also learn IN acoustic structure in the same way as motif syllables from a tutor. Since LMAN is known to be necessary for learning the acoustic structure of motifs, one would expect its lesion to affect IN learning as well. I quantified the acoustic structural similarity of the pupil's IN with its tutor using a software called *Sound Analysis Pro* (symmetric, time course similarity measurement using SAP2011, <http://soundanalysispro.com/>; Tchernichovski et al., 2000). The lesioned birds were divided into two groups – those with LMAN lesions and those with sham lesions – depending on the coordinates used for lesioning. As a control, I analyzed IN similarity data from socially tutored

birds that were tutored by a social tutor without any surgical intervention. IN similarity measurements could only be done for a subset of birds ($n = 7/21$) because, in some cases, either the tutor or the pupil was a no IN bird. In others, the pupil's song was too variable, and INs could not be defined. The IN acoustic similarity for the LMAN lesioned birds ($n = 5$) was found to be much lower compared to the sham lesioned birds ($n = 2$) and socially tutored birds ($n = 6$) (Wilcoxon rank sum test for group 1-3, $p = 0.0553$) (Figure 3.13). This suggests that the LMAN lesioned birds did not learn the acoustic structure of IN from their tutor.

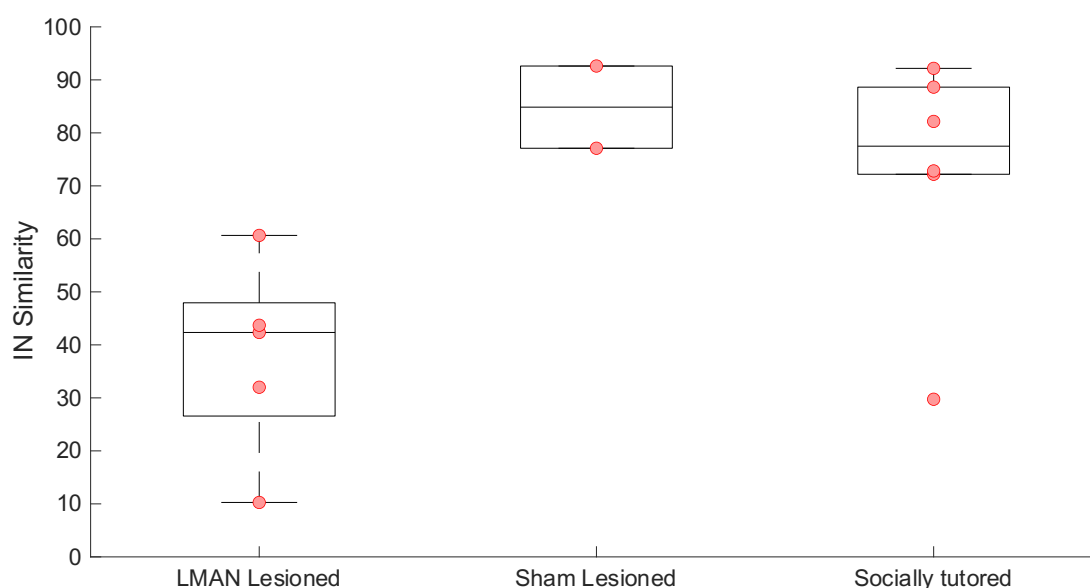


Figure 3.13: IN similarity of lesioned birds versus socially tutored birds.

Each point on the graph represents the average IN similarity for a given bird (greater than or equal to 60% lesioned ($n = 2$), less than 60% lesioned ($n = 1$), socially tutored ($n = 6$)) with its tutor. Socially tutored birds' data: Shikha Kalra

3.3.6 INs and Motifs are learned independently of each other

INs can be learned by a pupil in a way similar to motif syllables from the tutor. Hence, it is reasonable to anticipate a correlation between IN similarity and motif similarity of tutor and tutored birds. In order to check this, I compared the acoustic similarity of IN and motif for a given pupil with that of its tutor ($n = 7$). Although, I did not find any correlation between motif similarity and IN similarity of tutor and pupil (Pearson Correlation Coefficient, $r = -0.0201$, $p = 0.9658$) (Figure 3.14). Overall, this suggests that INs and motifs are learned independently of each other.

3.4 LMAN may influence learning of IN number

IN number is another property that the pupil can learn from its tutor. Hence, I wanted to check if LMAN is involved in learning of IN number as well. In order to do this, I compared the mean IN number of the pupil to that of its tutor. The lesioned birds were divided into two groups – those with LMAN lesions and those with sham lesions – depending on the coordinates used for lesioning. For reference, I compared IN number data from socially tutored pupils who were tutored by a social tutor without any surgical intervention.

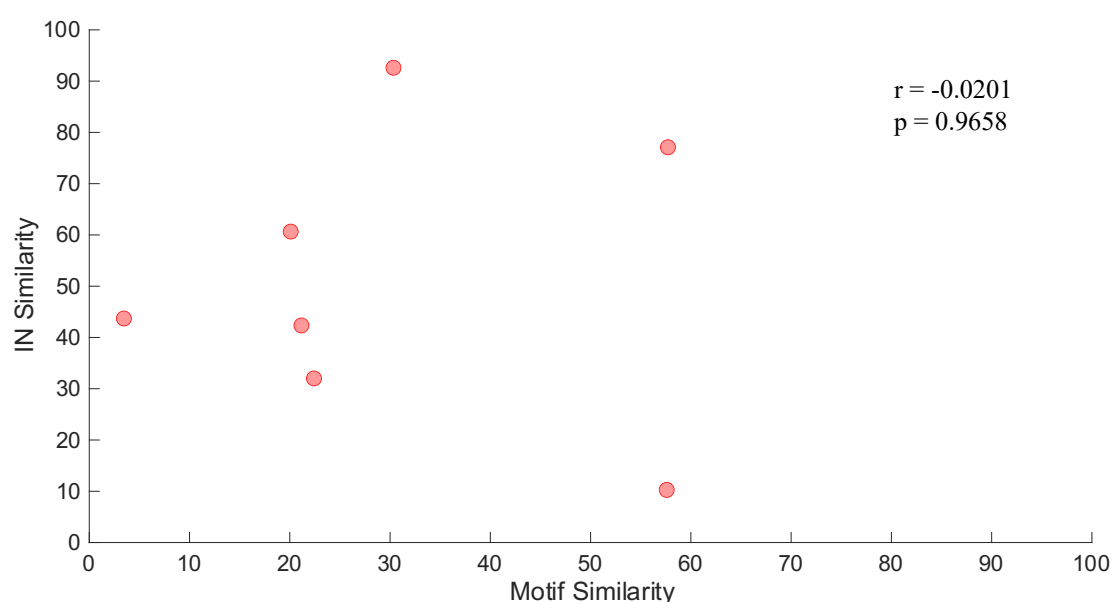


Figure 3.14: IN similarity versus motif similarity for lesioned birds.

Each point on the graph represents the average acoustic similarity values for a given pupil with its tutor ($n = 7$). Pearson Correlation Coefficient, $r = -0.0201$, $p = 0.9658$

There was no correlation between the mean IN number of LMAN lesioned pupils and its tutor ($n = 12$, Pearson Correlation Coefficient, $r = -0.1920$, $p = 0.5501$) (Figure 3.15 A). There may not be a correlation between the mean IN number of sham lesioned pupils and its tutor ($n = 7$, Pearson Correlation Coefficient, $r = -0.2638$, $p = 0.5675$), although this outcome is mainly influenced by an extreme outlier (Figure 3.15 B). Nevertheless, there seems to be a trend. Birds tutored with a tutor having low IN number learned songs starting with fewer number of INs, while birds tutored with a tutor having high IN number learned songs starting with higher number of INs. However, I need more sham lesioned birds to draw any firm conclusions. For socially tutored birds, there is a positive correlation between the mean IN number of tutor and

pupil ($n = 10$, Pearson Correlation Coefficient, $r = 0.5731$, $p = 0.0833$) (Figure 3.15 C). Overall, learning number of INs by the pupil from the tutor may be influenced by LMAN.

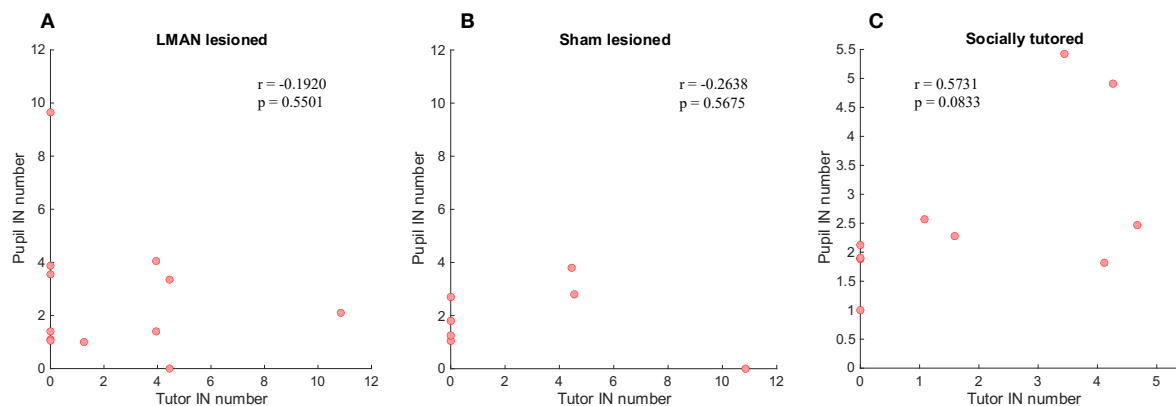


Figure 3.15: Mean IN number for tutor versus pupil.

Each point on the graph represents the mean IN number of pupils and its corresponding tutor. A) compares the mean IN number of LMAN lesioned pupils to that of their tutor ($n = 12$, Pearson Correlation Coefficient, $r = -0.1920$, $p = 0.5501$), (B) compares the mean IN number of sham lesioned pupils to that of their tutor ($n = 7$, Pearson Correlation Coefficient, $r = -0.2638$, $p = 0.5675$), and (C) compares the mean IN number of socially tutored pupils to that of their tutor ($n = 10$, Pearson Correlation Coefficient, $r = 0.5731$, $p = 0.0833$).

CHAPTER 4: Discussion

Children learn speech by imitating the vocalization of their parents. Apart from humans, other animals, such as birds, use vocal communication. Some songbirds learn songs from their father, and the zebra finch, an Australian songbird, is a well-studied example for vocal learning and production. Previous studies suggest that inputs from AFP are important for learning of songs in juvenile zebra finches, and any damage to this region during the song learning phase will significantly impede the normal progression of song learning (Bottjer et al., 1984; Scharff and Nottebohm, 1991). For a long time, people believed that only the motif part of the song is learned by the juvenile zebra finches. The INs in the zebra finch song were merely looked at as an introductory gesture and not much was known about its origin and function. In 2021, Kalra et al. showed that zebra finches can learn the introductory elements from a tutor just like any other syllable in the motif. Here, I have looked at whether LMAN, the output nucleus of AFP, has any role in learning specific properties of INs. Since INs also have a learned component just like any other syllable of the motif, and LMAN affects learning of motif syllables, I hypothesize that LMAN might have a role in learning specific properties of INs, specifically the number and acoustic structure.

4.1 Standardized LMAN coordinates, lesion parameters, and staining method

My initial focus was to reliably target LMAN and determine what lesion parameters to use to damage LMAN. I performed antidromic stimulation and fluorescent retrograde tracer injection in RA to locate LMAN. Next, I delineated the extent of LMAN and standardized the lesion parameters by performing anesthetized neural recordings followed by electrolytic lesions and histology. After that, I went on to perform bilateral electrolytic lesions on juvenile birds during the song learning phase.

I have tried multiple staining techniques, including IHC, to stain and visualize LMAN, but those protocols were not replicable (see 2.7.1 and 2.7.2 in Methods). Finally, I standardized a cresyl violet staining protocol for visualizing LMAN. This protocol worked consistently, and LMAN could be visualized in sagittal brain sections of lesioned birds. I finished histology from $n = 8/24$ pupils after they crossed post hatch day 120. My results show that $n = 6/7$ LMAN lesioned pupils have an average lesion $> 60\%$ across both hemispheres, and there is barely any reduction in the volume of LMAN for the sham lesioned bird ($n = 1$) (Figure 3.8). This suggests

that the coordinates and the lesion parameters used were precise and large enough to cover most of LMAN, and those used for sham lesions did not damage the LMAN nucleus.

4.2 *LMAN influences learning the number and structure of INs*

I have analyzed the final song recorded after post hatch day 120 from $n = 21/24$ pupils. Similar to previous studies, my results also suggest that lesions of LMAN during the song learning phase affect the accuracy of learning of motifs by the pupil. Qualitatively and quantitatively, the song of the LMAN lesioned bird is different from that of their tutor. In contrast, the song of the sham lesioned bird is similar to that of their tutor (Figures 3.9 and 3.10). The percentage motif similarity of LMAN lesioned pupils to their tutor is much lower than that observed in sham lesioned pupils and socially tutored pupils to their tutor (Figure 3.11). Birds with more fraction of LMAN lesioned learned poorer compared to sham lesioned birds. Unilateral lesions of LMAN may also affect song learning by the juveniles (Figure 3.12). However, I need data from more birds to have a firm conclusion. Similar to syllables in a motif, learning the acoustic structure of INs is also influenced by LMAN. The similarity in the acoustic structure of INs of LMAN lesioned pupils with their tutor is much lower than that between the sham lesioned pupils and the socially tutored pupils with their tutors (Figure 3.13). This suggests that LMAN is essential for learning the acoustic structure of INs by the pupil from a tutor just like any syllable in a motif. Interestingly, the acoustic structure INs and motifs are learned independently of each other (Figure 3.14). There is no correlation between IN learning and motif learning. Some birds learn the acoustic structure of INs well, whereas some others learn the acoustic structure of motifs.

The number of INs produced before the song is another property a pupil can learn from a tutor. In LMAN lesioned pupils, there is no correlation between the mean IN number of tutor and pupil. Conversely, socially tutored pupils show a positive correlation between the mean IN number of tutor and pupil. This suggests that LMAN lesioned birds did not learn the mean IN number from their tutor, whereas socially tutored birds did learn from their tutor in this aspect. Sham lesioned birds similarly exhibit no correlation between the mean IN number of tutor and pupil. However, this outcome is greatly influenced by an extreme outlier, and hence, I need more sham lesioned birds to reach a conclusive result.

These results imply that LMAN is crucial for learning songs by the pupil from a tutor. Similar to how lesions of LMAN affect learning of syllables in a motif, it also affects learning of the various properties of INs. Lesioning LMAN in a juvenile male finch during the song learning phase affects the accuracy of copying the number and the acoustic structure of INs by the pupil from a tutor. This suggests that the neural pathways responsible for learning of motifs is also controlling the learning of INs.

4.3 *Songs of some lesioned pupils stereotyped after post hatch day 90*

At post hatch day 90, some lesioned pupils started their song with variable vocalization that can neither be defined as IN nor motif. The songs recorded after post hatch day 120 from most of these birds were more stereotyped, and the song can be defined into INs and motifs. Most of these birds dropped the variable element in the beginning, and the INs became more evident after post hatch day 120 (Figure 4.1). Therefore, I used the post hatch day 120 song from LMAN/sham lesioned pupil for all the analyses. Interestingly, the socially tutored birds learn to produce songs that can be differentiated into INs and motifs by post hatch day 90 with a high similarity score with their tutor. In other words, the lesioned pupils took a longer time to produce a stereotyped song compared to the socially tutored birds, and this may be partially due to the stress from the lesion surgery during the song learning phase.

4.4 *Some sham lesioned birds did not learn the tutor's song*

A couple of the sham lesioned birds did not learn songs from their tutor. The song spectrograms from these pupils look qualitatively different from their tutor (Figure 4.2). A plausible reason for this could be that I might have damaged the axons from LMAN to RA while performing the sham lesion. In such cases, the cresyl violet stained sections would look like a sham lesioned bird because the LMAN nucleus is intact and the area of LMAN remaining might be similar to a control bird. But if the neural projections from LMAN to RA are damaged, then its behavior might be similar to that of an LMAN lesioned bird. Hence, cresyl violet staining may not work in such cases. Instead, performing IHC or injecting a fluorescent retrograde tracer would be more helpful in those particular cases. IHC with anti-calcitonin-gene-related-peptide antibody (CGRP) will stain for neurons in LMAN and their terminals in RA if the projections to RA are intact. Similarly, when a fluorescent retrograde tracer is injected in RA, LMAN will only show

fluorescence if active projections exist from LMAN to RA (Figure 2.10). Further, the projections coming to RA from LMAN can be visualized in fluorescent tracer injected sections (Figure 4.3).

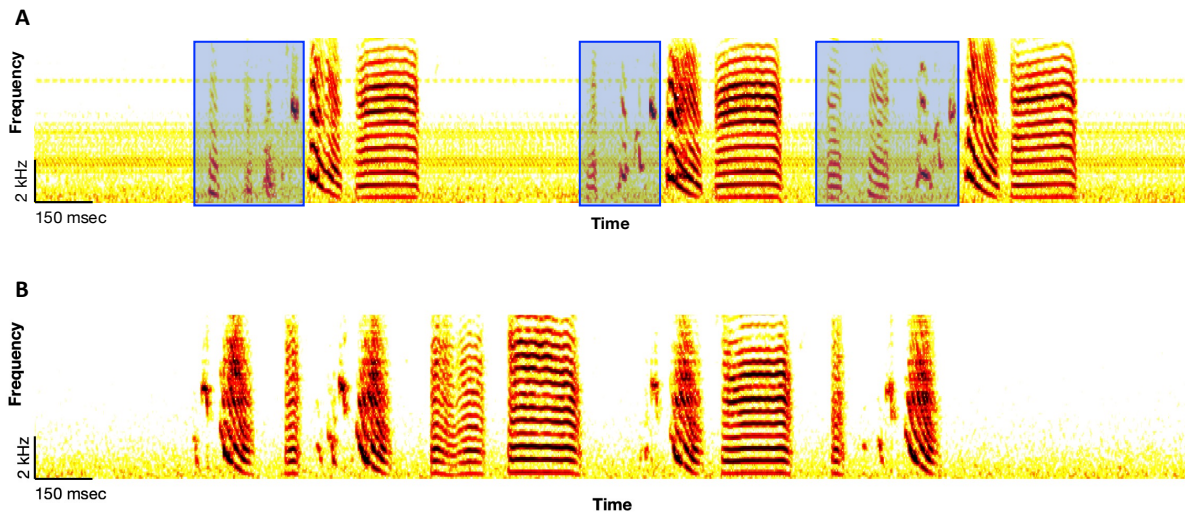


Figure 4.1: Song spectrogram of LMAN lesioned tutee

A) shows the song spectrogram of a tutee recorded after post hatch day 90, where the tutee begins the song with some variable vocalization (highlighted in blue). B) shows the song spectrogram of the same tutee recorded after post hatch 120, where the tutee no longer produces the variable vocalizations at the beginning of the song. Scale bar in (A) and (B): 150 msec (x-axis) and 2kHz (y-axis)

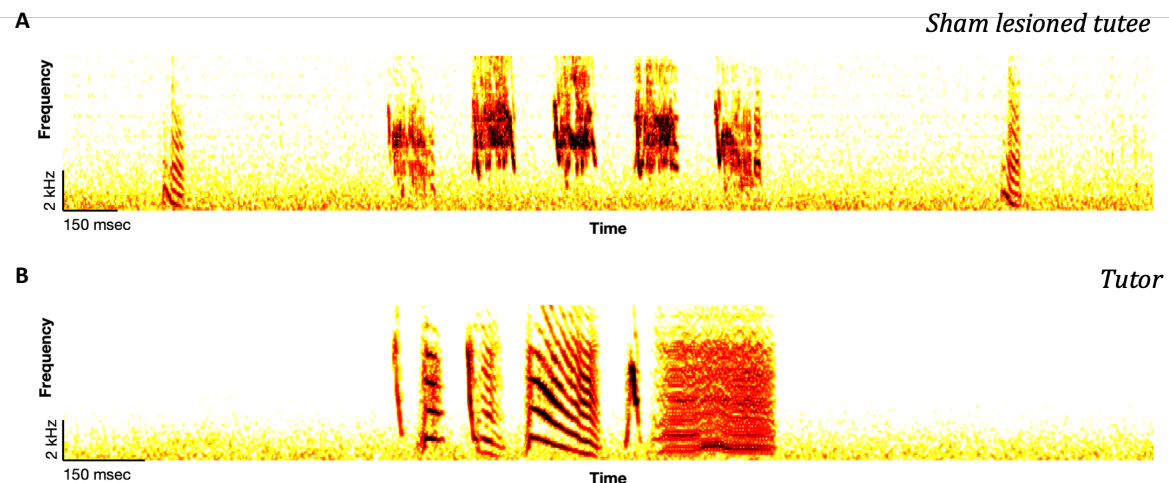


Figure 4.2: Song spectrogram of sham lesioned tutee

A) shows the song spectrogram of a sham lesioned tutee recorded on post hatch day 90 where the tutee did not learn the song from the tutor, and (B) shows the song spectrogram of the tutor. Scale bar in (A) and (B): 150 msec (x-axis) and 2kHz (y-axis)

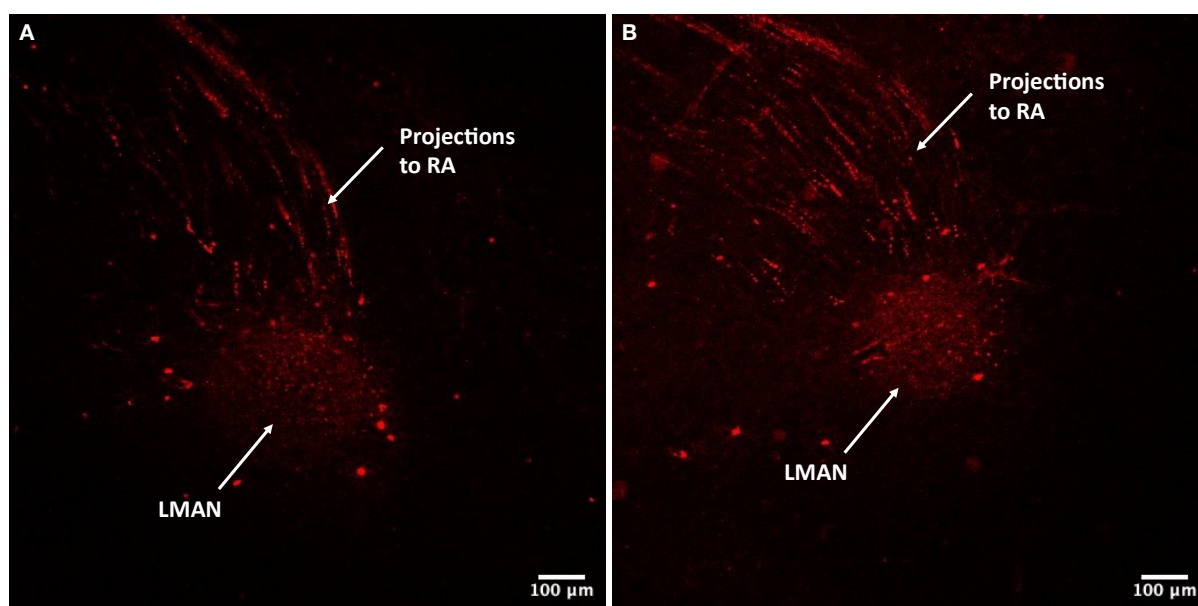


Figure 4.3: Confocal images of LMAN showing projections to RA.

A) and (B) show the confocal images of LMAN with its axons projecting to RA from a bird where I injected a fluorescent retrograde tracer in RA.

Future direction

The major setback with the project now is the number of birds for which the lesion size has been quantified. I have finished histology for $n = 9/24$ birds, and the lesion size has been successfully quantified for $n = 8/24$ birds. For one bird (blue115 left hemisphere), staining did not work properly, and hence, I had to skip this bird for all further analysis. Once the histology for all the birds is done, the volume of LMAN remaining can be quantified, and further analysis can be completed by comparing the volume of LMAN remaining with the accuracy of song copying.

Further, a few more birds ($n = 1$ LMAN and $n = 2$ sham) are yet to cross post hatch day 120. Once they cross this age, their songs can be recorded, and only then these birds can be included for further analysis. Next, I want to standardize a reliable way to visualize the damages to neuron projections. This can be done either by fluorescent retrograde tracer injections, or IHC, or by silver staining.

References

Bottjer, SW, Miesner, EA, and Arnold, AP (1984). Forebrain Lesions Disrupt Development But Not Maintenance of Song in Passerine Birds. *Science* 224, 901–903.

Brainard, MS (2004). Contributions of the Anterior Forebrain Pathway to Vocal Plasticity. *Annals of the New York Academy of Sciences* 1016, 377–394.

Byrne, RW, and Russon, AE (1998). Learning by imitation: A hierarchical approach. *Behav Brain Sci* 21, 667–684.

C, S, and F, N (1991). A comparative study of the behavioral deficits following lesions of various parts of the zebra finch song system: implications for vocal learning. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience* 11.

Doupe, AJ, and Kuhl, PK (1999). BIRDSONG AND HUMAN SPEECH: Common Themes and Mechanisms. *Annu Rev Neurosci* 22, 567–631.

Eales, LA (1985). Song learning in zebra finches: some effects of song model availability on what is learnt and when. *Animal Behaviour* 33, 1293–1300.

Eales, LA (1989). The influences of visual and vocal interaction on song learning in Zebra finches. *Animal Behaviour* 37, 507–508.

Fee, MS, and Scharff, C (2010). The Songbird as a Model for the Generation and Learning of Complex Sequential Behaviors. *ILAR Journal* 51, 362–377.

Gale, SD, and Perkel, DJ (2010). Anatomy of a songbird basal ganglia circuit essential for vocal learning and plasticity. *Journal of Chemical Neuroanatomy* 39, 124.

Ganos, C, Ogrzal, T, Schnitzler, A, and Münchau, A (2012). The pathophysiology of echopraxia/echolalia: Relevance to Gilles De La Tourette syndrome. *Movement Disorders* 27, 1222–1229.

Hahnloser, RHR, Kozhevnikov, AA, and Fee, MS (2002). An ultra-sparse code underlies the generation of neural sequences in a songbird. *Nature* 419, 65–70.

- Huber, L, Range, F, Voelkl, B, Szucsich, A, Virányi, Z, and Miklosi, A (2009). The evolution of imitation: what do the capacities of non-human animals tell us about the mechanisms of imitation? *Phil Trans R Soc B* 364, 2299–2309.
- Immelmann Klaus. (1969). Song development in the zebra finch and other estrildid finches. *In Hinde R.A., Bird Vocalizations. Cambridge University. Press, Cambridge*, 64–74.
- Kalra, S, Yawatkar, V, James, LS, Sakata, JT, and Rajan, R (2021). Introductory gestures before songbird vocal displays are shaped by learning and biological predispositions. *Proc Biol Sci* 288, 20202796.
- Kalra, S., (2023). Understanding the origin and function of introductory vocalizations before male zebra finch song [Doctoral dissertation, IISER, Pune]. IISER Pune Srinivasa Ramanujan Library Digital Repository.
- Kao, MH, and Brainard, MS (2006). Lesions of an Avian Basal Ganglia Circuit Prevent Context-Dependent Changes to Song Variability. *Journal of Neurophysiology* 96, 1441–1455.
- Kao, MH, Doupe, AJ, and Brainard, MS (2005). Contributions of an avian basal ganglia–forebrain circuit to real-time modulation of song. *Nature* 433, 638–643.
- Lomber, SG (1999). The advantages and limitations of permanent or reversible deactivation techniques in the assessment of neural function. *Journal of Neuroscience Methods* 86, 109–117.
- Nixdorf-Bergweiler, BE, Bischof, H-J, Nixdorf-Bergweiler, BE, and Bischof, H-J (2007). A Stereotaxic Atlas Of The Brain Of The Zebra Finch, *Taeniopygia guttata*, National Center for Biotechnology Information (US).
- Nottebohm, F, Stokes, TM, and Leonard, CM (1976). Central control of song in the canary, *Serinus canarius*. *J of Comparative Neurology* 165, 457–486.
- Ölveczky, BP, Andalman, AS, and Fee, MS (2005). Vocal Experimentation in the Juvenile Songbird Requires a Basal Ganglia Circuit. *PLoS Biol* 3, e153.
- Price, PH (1979). Developmental determinants of structure in zebra finch song. *Journal of Comparative and Physiological Psychology* 93, 260–277.

- Roper, A, and Zann, R (2006). The Onset of Song Learning and Song Tutor Selection in Fledgling Zebra Finches. *Ethology* 112, 458–470.
- Schindelin, J, Arganda-Carreras, I, Frise, E, Kaynig, V, Longair, M, Pietzsch, T, Preibisch, S, Rueden, C, Saalfeld, S, Schmid, B, *et al.* (2012). Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9, 676–682.
- Slater, PJB, Eales, LA, and Clayton, NS (1988). Song Learning in Zebra Finches (*Taeniopygia guttata*): Progress and Prospects. In: *Advances in the Study of Behavior*, Elsevier, 1–34.
- Sohrabji, F, Nordeen, EJ, and Nordeen, KW (1990). Selective impairment of song learning following lesions of a forebrain nucleus in the juvenile zebra finch. *Behavioral and Neural Biology* 53, 51–63.
- Sossinka, R, and Böhner, J (1980). Song Types in the Zebra Finch *Poephila guttata castanotis*.¹ *Zeitschrift Für Tierpsychologie* 53, 123–132.
- Stepanek, L, and Doupe, AJ (2010). Activity in a Cortical-Basal Ganglia Circuit for Song Is Required for Social Context-Dependent Vocal Variability. *Journal of Neurophysiology* 104, 2474–2486.
- Tchernichovski, O, Nottebohm, F, Ho, CE, Pesaran, B, and Mitra, PP (2000). A procedure for an automated measurement of song similarity. *Animal Behaviour* 59, 1167–1176.
- Vates, GE, and Nottebohm, F (1995). Feedback circuitry within a song-learning pathway. *Proc Natl Acad Sci U S A* 92, 5139–5143.
- Zann, RA (1996). *The zebra finch: a synthesis of field and laboratory studies*, Oxford ; New York: Oxford University Press.

Appendix

The following figures show the spectrograms of songs recorded from the pupil after post hatch day 120 and that of their tutors. The type of lesion (LMAN/sham) is mentioned against each tutee's name. Sections 2.8.2 and 2.8.3 in methods explains how average IN number and the percentage similarity scores were calculated. Scale bar in all the graphs: 150 msec (x-axis) and 2kHz (y-axis)

Tutee: blue114 (sham)

Tutor: orange19black199

% Similarity

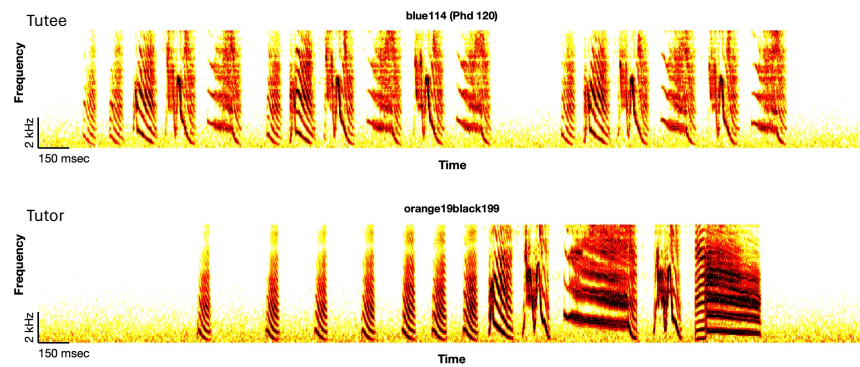
IN = 77.10

Motif = 57.71

Avg IN No.

Tutor: 4.55

Pupil: 2.80



Tutee: orange109 (sham)

Tutor: red084blue28

% Similarity

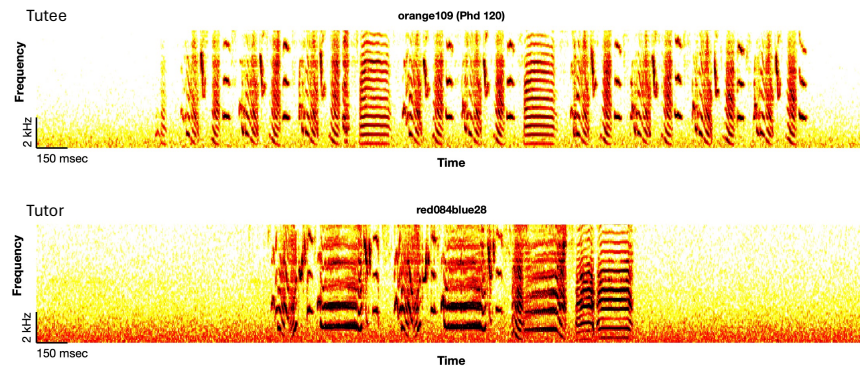
IN = N.A.

Motif = 21.69

Avg IN No.

Tutor: 0

Pupil: 1.05



Tutee: orange129 (sham)

Tutor: green42blue24

% Similarity

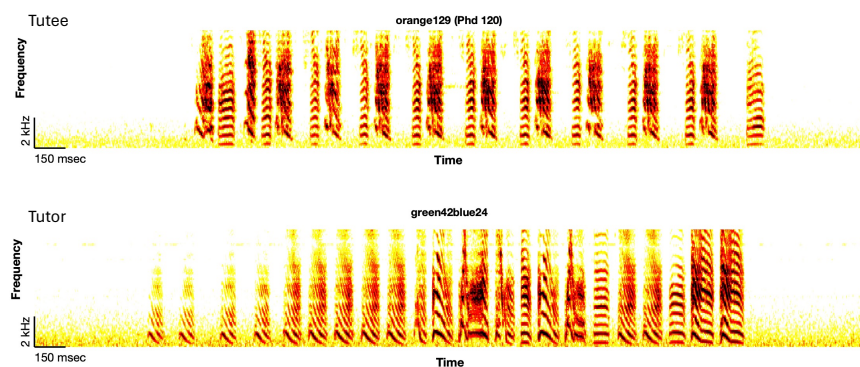
IN = N.A.

Motif = 48.01

Avg IN No.

Tutor: 10.85

Pupil: 0



Tutee: orange135 (sham)

Tutor: green107white111

% Similarity

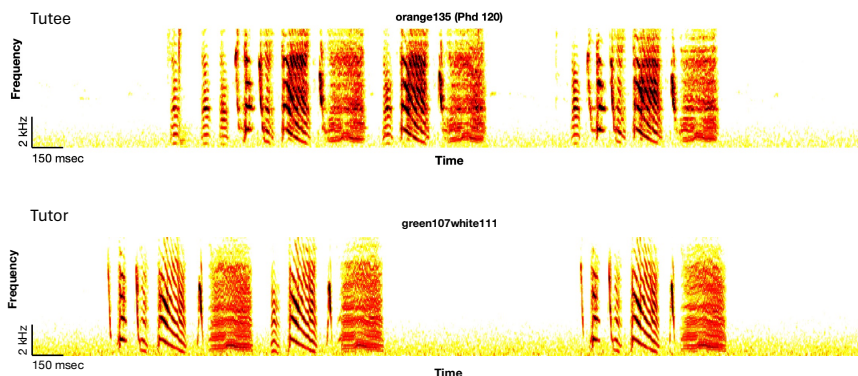
IN = N.A.

Motif = 86.42

Avg IN No.

Tutor: 0

Pupil: 2.70



Tutee: red190 (sham)

Tutor: blue95red107

% Similarity

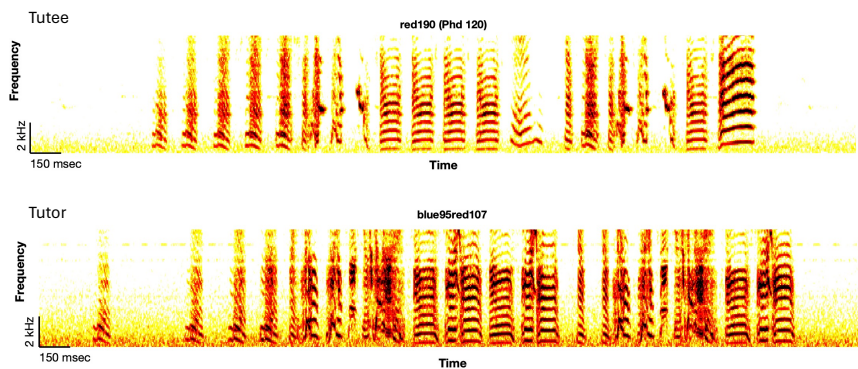
IN = 92.61

Motif = 30.34

Avg IN No.

Tutor: 4.45

Pupil: 3.80



Tutee: yellow143 (sham)

Tutor: green152white160

% Similarity

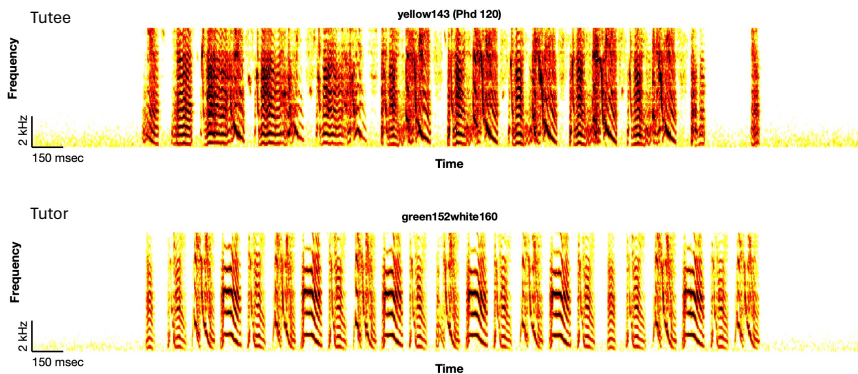
IN = N.A.

Motif = 43.12

Avg IN No.

Tutor: 0

Pupil: 1.25



Tutee: yellow144 (sham)

Tutor: green107white111

% Similarity

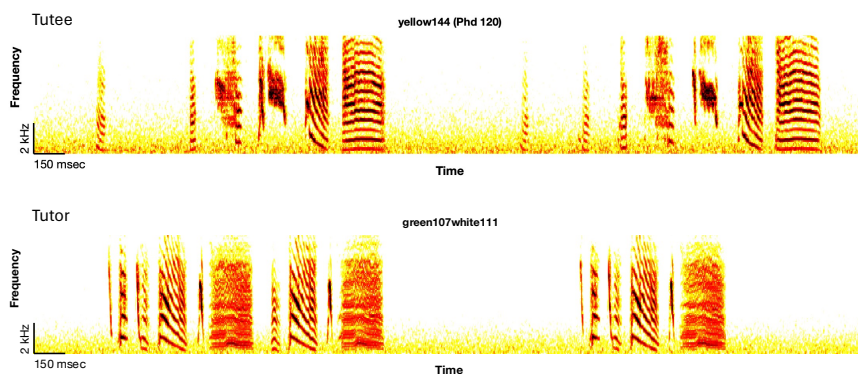
IN = N.A.

Motif = 44.12

Avg IN No.

Tutor: 0

Pupil: 1.80



Tutee: blue113 (LMAN)

Tutor: blue95red107

% Similarity

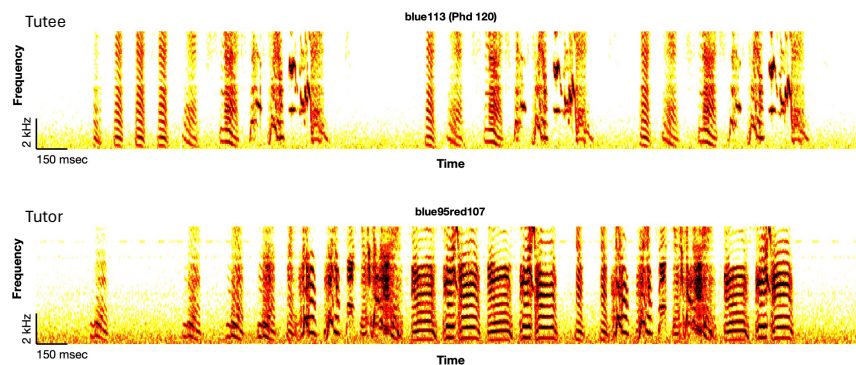
IN = 10.27

Motif = 57.59

Avg IN No.

Tutor: 4.45

Pupil: 3.35



Tutee: blue115 (LMAN)

Tutor: green125green126

% Similarity

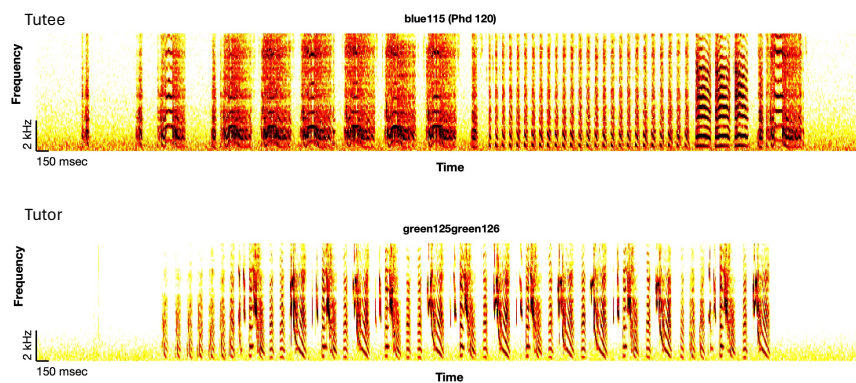
IN = 32.01

Motif = 22.41

Avg IN No.

Tutor: 3.95

Pupil: 1.40



Tutee: blue126 (LMAN)

Tutor: black120

% Similarity

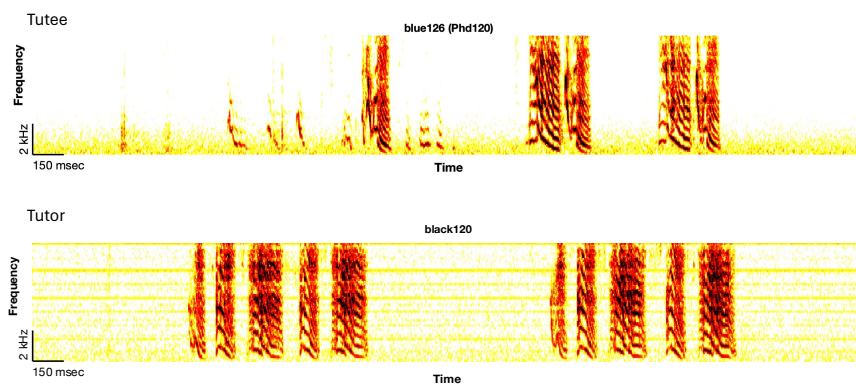
IN = N.A.

Motif = 39.30

Avg IN No.

Tutor: 0

Pupil: N.A.



Tutee: blue172 (LMAN)

Tutor: brown49orange49

% Similarity

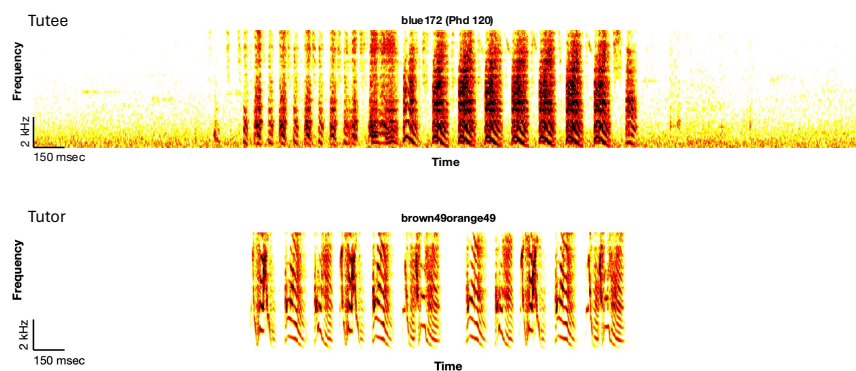
IN = N.A.

Motif = 5.83

Avg IN No.

Tutor: 0

Pupil: 9.65



Tutee: blue173 (LMAN)

Tutor: red084blue28

% Similarity

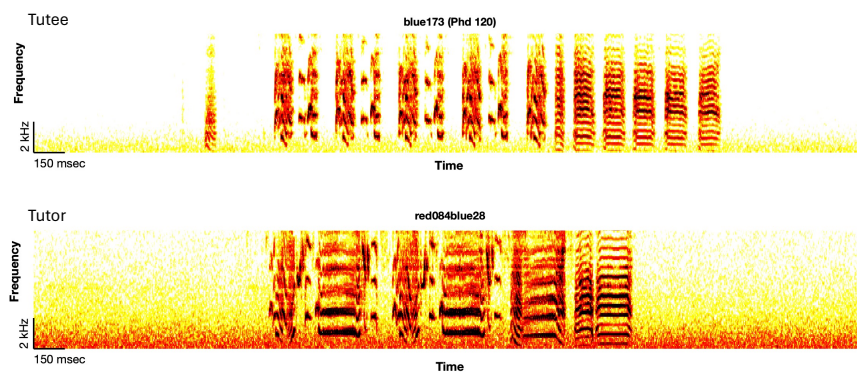
IN = N.A.

Motif = 27.90

Avg IN No.

Tutor: 0

Pupil: 1.40



Tutee: blue183 (LMAN)

Tutor: green42blue24

% Similarity

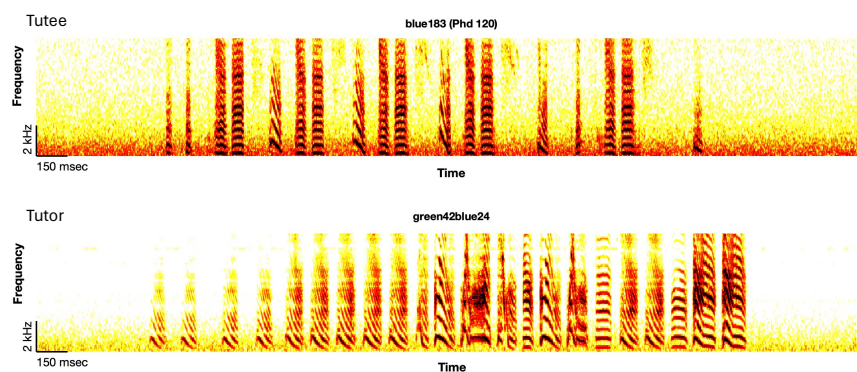
IN = 60.65

Motif = 20.09

Avg IN No.

Tutor: 10.85

Pupil: 2.10



Tutee: orange110 (LMAN)

Tutor: brown49orange49

% Similarity

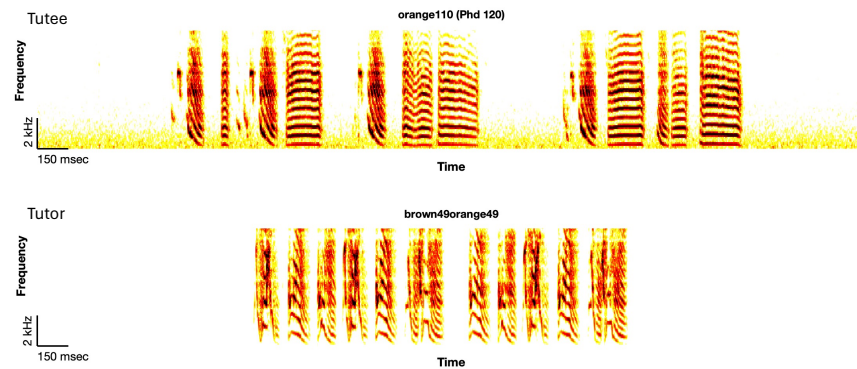
IN = N.A.

Motif = 9.74

Avg IN No.

Tutor: 0

Pupil: 1.10



Tutee: orange130 (LMAN)

Tutor: green192white155

% Similarity

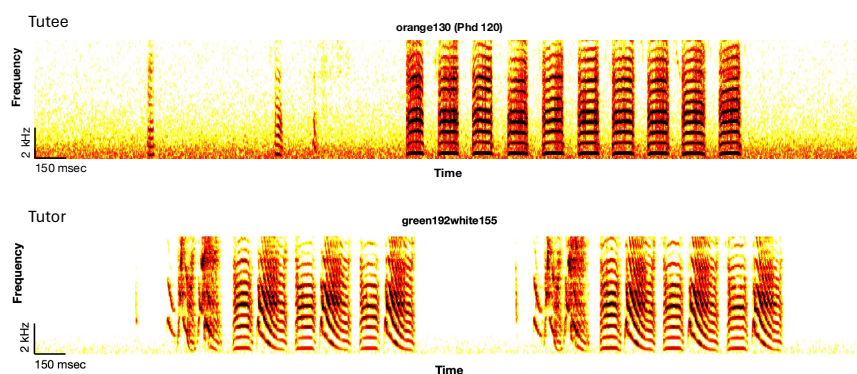
IN = 43.70

Motif = 3.45

Avg IN No.

Tutor: 1.25

Pupil: 1.00



Tutee: orange134 (LMAN)

Tutor: green152white160

% Similarity

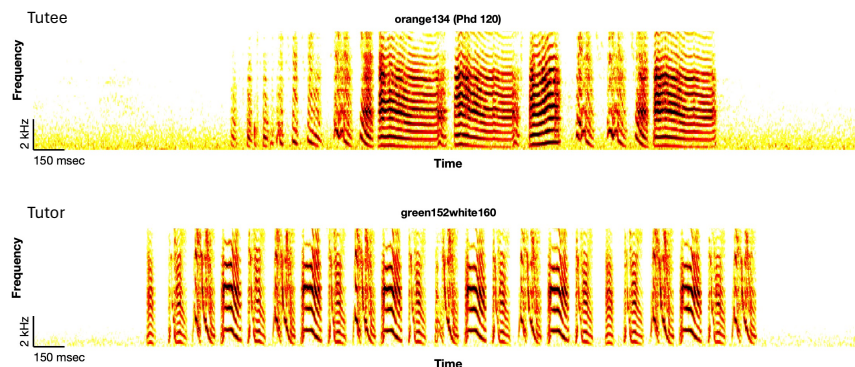
IN = N.A.

Motif = 35.46

Avg IN No.

Tutor: 0

Pupil: 3.55



Tutee: pink164 (LMAN)

Tutor: black120

% Similarity

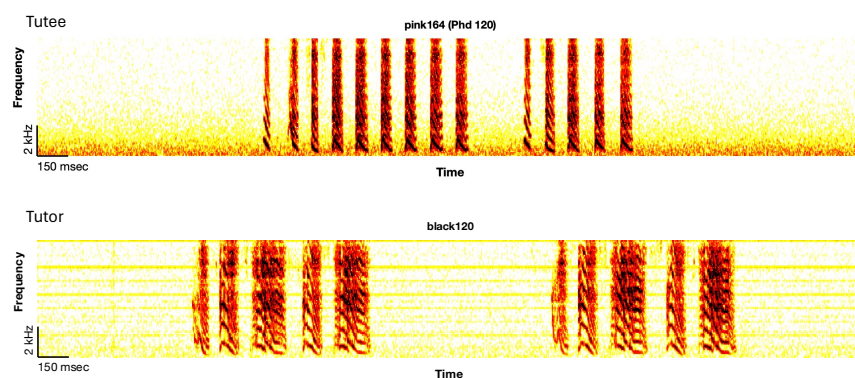
IN = N.A.

Motif = 29.25

Avg IN No.

Tutor: 0

Pupil: 1.05



Tutee: red134 (LMAN)

Tutor: blue95red107

% Similarity

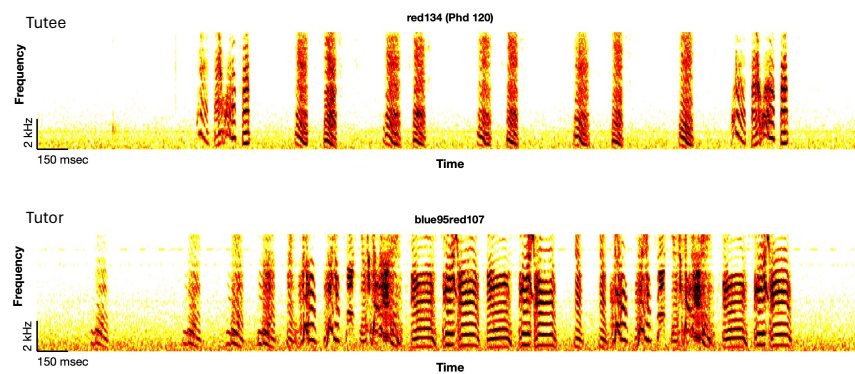
IN = N.A.

Motif = 37.53

Avg IN No.

Tutor: 4.45

Pupil: 0



Tutee: red140 (LMAN)

Tutor: yellow74brown01

% Similarity

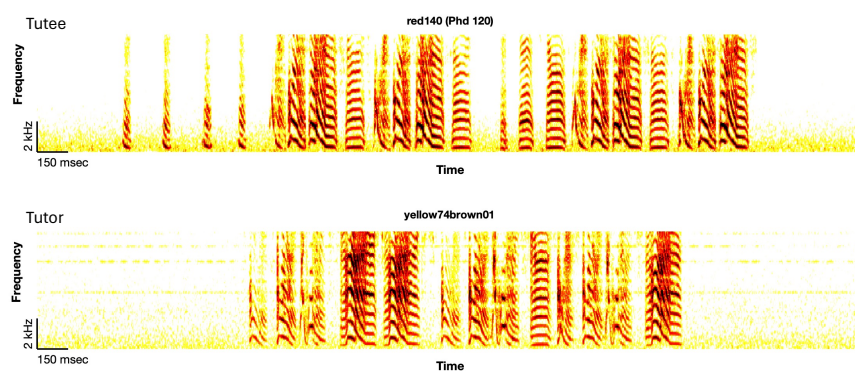
IN = N.A.

Motif = 32.91

Avg IN No.

Tutor: 0

Pupil: 3.88



Tutee: red179 (LMAN)

Tutor: green125green126

% Similarity

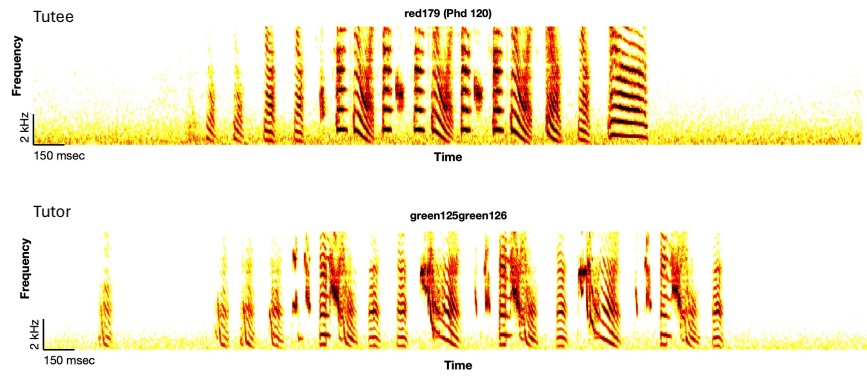
IN = 42.34

Motif = 21.16

Avg IN No.

Tutor: 3.95

Pupil: 4.05



Tutee: yellow141 (LMAN)

Tutor: green192white155

% Similarity

IN = N.A.

Motif = 43.95

Avg IN No.

Tutor: 1.25

Pupil: N.A.

