

Understanding the origin and function of introductory vocalizations before male zebra finch song

विद्या वाचस्पति की
उपाधि की अपेक्षाओं की आंशिक पूर्ति में प्रस्तुत शोध प्रबंध

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DECLARATION

I declare that this written submission represents my ideas in my own words and where other's ideas have been included; I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.



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CERTIFICATE

Certified that the work incorporated in the thesis entitled “**Understanding the origin and function of introductory vocalizations before male zebra finch song**” submitted by **Ms. Shikha Kalra** (Reg. No. 20142005) was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other university or institution.



Dr. Raghav Rajan
Supervisor

Date: 6th October, 2023

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Synopsis

Introduction

Many animals start their communicative displays with introductory gestures. Introductory gestures/introductory notes precede both learned and innate displays. Some examples of animals that perform introductory gestures include lizards, which do introductory pushups/tail flicks before their head bobbing display (Fleishman, 1992; Peters & Evans, 2003); many songbirds start their song with introductory notes (Chelén et al., 2005; Kroodsma et al., 1987; Price, 1979; Richards, 1981; Soha & Marler, 2000; Sossinka & Böhner, 1980; Zann, 1996); frogs (Backwell, 1988) and toadfish (Thorson & Fine, 2002) produce introductory notes before their aggressive/advertisement calls. Different functions have been suggested for introductory gestures including, an altering component before the main message in songbirds (Richards, 1981) and lizards (Ord & Stamps, 2008; Richards, 1981); male-male competition in frogs (Littlejohn & Harrison, 1985); species-song recognition by juvenile in some songbird species for copying the correct song (Soha & Marler, 2000) and motor preparation in another songbird species, the zebra finch (Rajan & Doupe, 2013). However, the origin and properties of these introductory gestures are not fully understood. In my thesis, I have used the zebra finch as a model system to address some of these questions. The zebra finch is a well-studied Australian desert songbird (Zann, 1996). In this species, only males sing as a part of a courtship display. These birds learn the song from the tutor, in most cases its father during the first 90 days after hatching. Song does not change after this (Immelmann Klaus, 1969). The song of zebra finch consists short-duration repeating vocalizations called introductory notes (INs) and a consistent sequence of vocalizations called motif/song. INs are variable in number across song bouts (Price, 1979; Sossinka & Böhner, 1980; Zann, 1996). The presence of INs before learned song makes the zebra finch a good model system to study the origin and function of INs in the context of learned vocalizations. In order to understand the origin and function of INs, I have looked at the following four aims in my thesis.

Aim 1 (Chapter 2): Understanding the origin of introductory notes.

Previous studies have shown that young zebra finches learn song from their father (Immelmann Klaus, 1969). However, it is not known if INs are also learned. In this chapter, I have used different tutoring methods to examine the origin of INs. Firstly, by comparing the properties of INs (mean IN number and acoustic structure) between father and pupil, I have shown that they are similar. This similarity could arise because the juvenile inherits it from the father, or it can

be due to learning. In order to distinguish between the two, I have used different tutoring methods, namely 1) social tutoring and 2) playback tutoring. In the case of social tutoring, naive male juvenile zebra finches were tutored by another adult zebra finch (tutor) whose INs are different from that of its father. These birds developed INs similar to the tutor rather than the father. Playback tutored birds were artificially tutored with zebra finch song without INs, played back through a speaker. Even though these birds were not exposed to INs, they initiated song with INs. However, the INs produced by these birds were different from their father. Further, similar to an earlier (Price, 1979), I have shown that birds never exposed to the song or INs during the song-learning phase also initiate song with INs. My results suggest that zebra finches have an innate template for initiating song with IN. Thus, INs, similar to the song, has both learned and innate component.

Aim 2 (Chapter 3): Characterize innate/learned properties of introductory notes.

In Chapter 2, In order to delineate and characterize the learned and innate properties of INs, I have compared properties of INs among different groups of birds, namely: 1) normally reared birds, 2) playback tutored birds, and 3) isolates (birds reared in the absence of exposure to the song). Here, I have shown that birds reared without exposure to INs also initiate song with INs. The mean number of times these birds repeat INs is in the same range as normally reared birds. Similar to the normally reared birds, INs produced by birds reared without exposure to INs are shorter in duration compared to the motif syllables. Further, acoustic properties of INs, like mean frequency, frequency modulation, and duration, are similar across groups. At the same time, properties like entropy, entropy variance, and pitch goodness are different. Next, by comparing the structural similarity of INs among isolates, I show that there is inter-individual variability in the structure of INs produced by isolates. Furthermore, the INs produced by isolates are not similar to innate vocalizations like calls. Interestingly, INs produced by birds reared without exposure to INs also show temporal and acoustic progression; i.e. the intervals between INs become shorter and more stereotyped and the acoustic structure of successive INs becomes more similar to the acoustic structure of the last IN. These properties have been demonstrated in birds with normal exposure to song and INs. Overall, properties of INs produced by birds reared in the absence of exposure to IN are similar to that of normally reared birds, but the presence of tutor influences properties like mean IN number and IN structure.

Aim 3 (Chapter 4): Brain region involved in the learning of introductory notes.

I have shown in Chapter 2 and 3 that INs, similar to song, have both learned and innate properties. Next, I asked if a brain region responsible for song learning is also involved in learning of INs. Different brain regions have been identified to be involved in song production and learning (Bottjer et al., 1989; Nottebohm et al., 1976). Anterior forebrain pathway (AFP) is involved in song learning (Bottjer et al., 1984; Goldberg & Fee, 2011; Scharff & Nottebohm, 1991; Sohrabji et al., 1990) and LMAN (lateral portion of the magnocellular nucleus of the anterior nidopallium) is the output nucleus of AFP (Bottjer et al., 1989). Lesioning LMAN in juvenile zebra finches during the song-learning phase affects the accuracy with which song is copied by the juvenile zebra finches (Bottjer et al., 1984; Scharff & Nottebohm, 1991). In order to check if LMAN is involved in learning INs as well, I have performed bilateral electrolytic lesions in juvenile zebra finches. I have shown that birds with 20-60% LMAN lesioned produce INs with lower structural similarity with tutor than birds with <20% lesion. In contrast, the mean IN number for both groups of birds seems similar to the tutor. However, many birds had incomplete lesions and so due to the small number of birds with lesions, it is difficult to draw a conclusion regarding the involvement of LMAN in learning of INs.

Aim 4 (Chapter 5): Understanding the function of introductory notes.

Different functions have been suggested for introductory notes in different songbird species (Rajan & Doupe, 2013; Richards, 1981; Soha & Marler, 2000). In zebra finches, INs have been suggested to be involved in motor preparation (Rajan & Doupe, 2013), but their role in communication is not known. It is known that zebra finches produce more introductory notes when singing to a female zebra finch (directed song) compared to when singing in the absence of a female (alone song) (Sossinka & Böhner, 1980). Further, it has been shown that female zebra finches prefer directed songs over alone songs (Woolley & Doupe, 2008). It is not known if female zebra finches have a preference for songs starting with INs as well. Here, I have played back different stimuli to check if female zebra finches prefer songs starting with INs. I have used the number of calls produced by the female as a measure of preference. Different stimuli used were 1) song starting with INs, 2) song without INs, 3) reverse song (song followed by INs), 4) only IN, and 5) noise. Birds responded equally to all stimuli except noise, suggesting that female zebra finches don't necessarily have a preference for song starting with INs. Adult male zebra finches increase the number of INs when directing their song to a juvenile (Chen et

al., 2016). This could be a way to ensure that the juvenile birds pay more attention to the song and could influence copying of song. However, I did not find any correlation between the number of INs produced by the male and the accuracy of song copying. Overall, my results suggest that females don't have preference for songs starting with INs, and the number of INs before the tutor's song doesn't influence the accuracy of song copying by the juvenile.

In Chapter 6, I summarize my results and discuss their implications and future directions. My results suggests that similar to song, INs also have learned and innate properties. Brain regions involved in the learning of songs might be involved in the learning of some IN features. Regarding the function of INs, female zebra finches don't prefer songs starting with IN under normal conditions. It is possible that similar to frogs, INs might have communicative functions in case of male-male competition. However, this needs to be tested. Overall, my thesis shows that INs, similar to the song, have both learned and innate components and my thesis also highlights the zebra finch as a good model to understand the properties and function of introductory gestures before complex animal displays.

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Chapter 1

Introduction

Communication involves the transfer of information from the sender to the receiver. The sender should be able to convey the information effectively to get the desired response. While, the receiver should be able to decode the message in the particular signal and choose to respond accordingly. This communication involves sensory modalities like auditory, visual, olfactory, tactile, or a combination of two or more (multisensory). Over the decades, animal/insect communication studies have shown that animals use these modalities for both heterospecific and conspecific communication (Bradbury & Vehrencamp, 2011).

1.1 Information conveyed using different sensory modalities

In order to survive, animals should be able to communicate information, like willingness to mate, availability of food, and presence of predators, effectively. They use sensory systems like visual, acoustic, olfactory, and tactile for communicating such information. For instance, peacocks and peacock spiders use the visual signal to communicate. Both peacocks and peacock spiders perform elaborate dances to woo the female. During the dance, the peacock displays its extravagant tail, and the peacock spider opens its opisthosomal flaps and uses sequential movement as part of the courtship display (Girard et al., 2011; Petrie et al., 1991). Another example of visual signals is bioluminescence, used by insects (reviewed in Lloyd, 1971). Fireflies are known to communicate using light flashes; different species of fireflies exhibit different flash patterns (SELIGER et al., 1964). Besides visual signals, animals also use acoustic signals to interact with conspecific and heterospecific with or without accompanying visual display. Examples of acoustic communication include calls produced by birds in different contexts (Zann, 1996). Communication also involves olfactory cues. Dogs use olfactory cues to mark their territories (Pal, 2003), while ants leave trails of chemicals for other ants to follow during foraging (Beckers et al., 1992), and honey bees mark flowers from which they extract nectar with chemicals (Cameron, 1981). Other modes of communication involve signals generated using vibration (Markl, 1983). Kangaroo rats perform foot drumming in response to predators like snakes in burrows (Randall, 1997; Randall & Stevens, 1987). Other than the modalities mentioned, electric fishes use electric discharge for communication (reviewed in Hopkins, 1988).

Irrespective of the sensory modality used for communication, animals have developed different signals for providing a range of information. Different animals can use the same sensory modality to convey different information or convey the same information using different sensory modalities. In order to show a willingness to mate and fitness, songbirds like zebra finches (Sossinka & Böhner, 1980) and drosophila (Ewing & Bennet-Clark, 1968) produce

courtship songs with/without accompanying visual display. Females use these displays to decipher male fitness and decide whether to mate. As mentioned before, to survive, animals also need to convey other information like the presence of a predator, the location of food, etc. Animals produce alarm calls to warn conspecific about the presence of a predator. While they give mobbing signals to scare the predator away, Japanese tits produce different mobbing calls for crows and snake predators (Suzuki & Ueda, 2013). To convey the location of food, ants use chemical signals (Beckers et al., 1992), while honey bees use visual signals (Frisch, 1993). This suggests that animals can produce different signals to effectively deliver their message. Next, an obvious question one would ask is if they are capable of modulating signals for effective communication. Studies have shown that animals can modify/combine signals to convey necessary information under different conditions.

1.2 Effect of environment and social context on signals

The reception efficiency of a signal will depend on the signal-to-noise ratio. High interference of noise from the surroundings lowers the signal-to-noise ratio; hence, the message is likely to be masked by noise. Thus, to convey the message effectively, animals must be able to modify the signal in real-time. This interference could be caused by environmental noise, like differences in light conditions in morning and evening (under dim light conditions, detection of visual signals can become difficult), or social context, like the distance from the receiver or the number of conspecifics around. Studies have shown that animals can assess the environment and modify the signals accordingly to maximize information transfer. The zebra finch, a songbird, can increase the amplitude of its song in the presence of background noise (Cynx et al., 1998), and frogs change their type of modality used for communication based on light conditions (Grafe & Wanger, 2007). Foot-flagging frogs perform more foot-flagging (Figure 1.1A) in the morning, while they produce more calls in the early morning when the light level is low (Grafe and Wanger, 2007). All this shows that animals can access their environment and modify signals accordingly for effective communication. In acoustic communication, the distance between the sender and receiver influences the amplitude with which the sender produces vocalization. Zebra finches (Australian desert song birds; male zebra finch sings as a part of courtship display (Zann, 1996)) can increase the amplitude of their song, and the type of song changes based on distance from the female zebra finch (Brumm & Slater, 2006; Suri & Rajan, 2018). Similarly, the distance from the receiver also influences visual signals, such as in fiddler crab. The fiddler crab (*Uca perplexa*) reduces the duration of

the claw waving display (Figure 1.1B) as the distance from the female reduces (How et al., 2008). Further, social context also influences the claw waving in fiddler crabs. They increase waving with females present compared to when females are absent (Pope, 2000). Other than assessing the environment and distance from the receiver to modify signals, animals can associate given vocalizations with a specific context. This association provides the receiver with more information, and thus it can react accordingly. One such example is the Japanese great tits. It produces different types of calls for different predator types, namely 'chicka' in response to crows, while 'jar' calls are given to snakes (Suzuki, 2014). Interestingly, nestlings recognize these vocalizations and their association. Nestlings crouch in response to playback of 'chicka' calls (crows take nestlings using their beak from the nest) and jump off from the nest in response to 'jar' calls (snakes crawl into the nest) (Suzuki, 2011). Similar to Japanese great tits, putty-nosed monkeys also produce different alarm calls for leopard and crowned eagle (Arnold & Zuberbühler, 2008). Vervet monkeys produce different alarm calls for terrestrial and aerial predators (Seyfarth et al., 1980). Another example is the zebra finch. They are also known to produce various calls in different contexts. They produce long calls in acoustic isolation, tet calls before take-off or any other movement, and they give distress calls when in contact with a potential predator (Zann, 1996). These suggest that animals can associate a simple vocalization with a given context. Further, studies have shown that they can combine these simple vocalizations into meaningful sentences (Arnold & Zuberbühler, 2008; Suzuki et al., 2016).

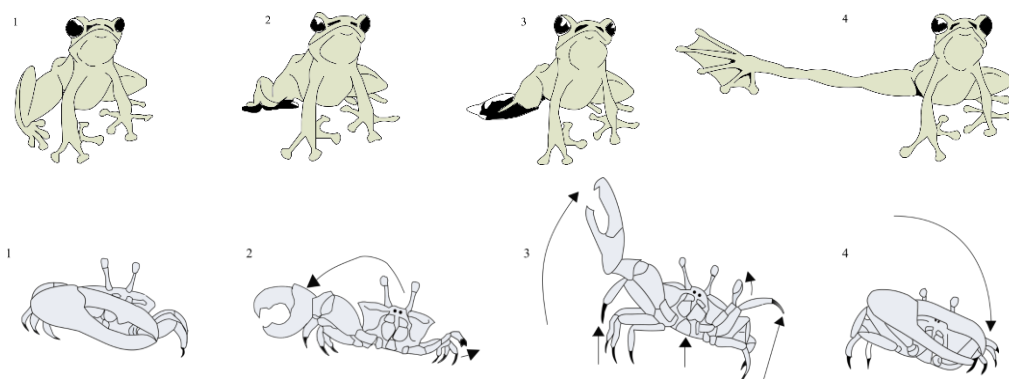


Figure 1.1 Foot flagging and claw waving sequence in frog and fiddler crab.

A. Foot flagging sequence for a foot flagging female frog (*Staurois guttatus*), Adapted and modified from Grafe and Wanger, 2007. B. Different stages of claw waving display by a fiddler crab (*Uca perplexa*), Adapted and modified from How et al., 2008

1.3 Combinatorial capacity in animals

Producing different kinds of calls for each context will require animals to have a large number of different calls in their repertoire. However, combining individual vocalizations increases the context for using a given set of calls. Studies have shown that animals can combine the different vocalizations in their repertoire to provide different information. Like humans, non-human primates also can combine different vocalizations based on the social context. Campbell's monkey can combine different calls to produce sequences used in both predatory and nonpredatory contexts. The male monkey gives a pair of 'boom' calls in the absence of visual contact with its group members. While it produces a pair of 'boom' calls followed by 'krak-oo' calls in response to a fight between other monkeys or a falling branch of a tree. Further, it does a sequence of 'krak-oo' calls upon hearing the sound of a predator (Ouattara et al., 2009). A sequence of 'hack' and 'pyows' calls given by putty-nosed monkeys predicts group movement, while it does individual calls in response to different predators (Arnold & Zuberbühler, 2008). Furthermore, in the case of Campbell's monkey, it has been shown that they add a suffix to the calls produced in response to a different predator for various ranges of disturbances (Coye et al., 2015; Ouattara et al., 2009). This capacity to combine vocalizations is not limited to primates; birds can also combine vocalization. Japanese tits produce a combination of calls 'ABC' in a predatory context and another vocalization, 'D' alone or combined with 'ABC.' Birds start to move towards the sound source with high horizontal scans when 'ABC-D' is played back, unlike playback to only 'ABC' (birds show high horizontal scans) or 'D' (less horizontal scans but approaches the speaker). However, birds do fewer horizontal scans and rarely approach the sound source in response to the playback 'D-ABC' sequence. This suggests they recognize the 'ABC-D' sequence differently from 'ABC' or 'D' (Suzuki et al., 2016). The ability to recognize this sequence of alert calls followed by a recruitment call was tested by playback of the Japanese tits alert call in combination with the recruitment call of willow tits. Japanese tit responded to this sequence similarly to a combination of 'ABC-D', suggesting the ability to recognize sequence (Suzuki et al., 2017). Other birds, like pied babbler, also produce a sequence consisting of an alert call followed by a recruitment call. Playback of this sequence increases attentiveness in babblers, and they tend to approach sound source faster than playback of only recruitment or alert calls (Engesser et al., 2016). Overall, this suggests that animals can combine simple vocalizations to form complex sequences based on the context.

1.4 The function of introductory gestures before the main display

In the examples mentioned above, each component of a complex sequence has individual meanings. However, some animals produce a sequence of display, where entire sequence has a specific meaning. Some examples of such sequences include songs produced by songbirds, visual displays by lizards, etc. Interestingly, these displays also consist of two parts: the first part, mostly the introductory portion (introductory gestures), and the second part, which contains the main message. Many animals, like songbirds (Price, 1979; Sossinka & Böhner, 1980; Zann, 1996), frogs (Backwell, 1988), lizards (Fleishman, 1992; Peters & Evans, 2003), toadfish (Thorson & Fine, 2002), and owls (Otter, 1996), have been shown to produce introductory gestures before their main messages. There are different functions speculated for these introductory elements in different animals. Introductory gestures have been hypothesized to perform different functions like information carriers during noisy conditions, conspecific identification signals, and motor preparation for the main message. In the following section, I will discuss studies to understand the function of introductory notes across different animals irrespective of the sensory modality involved in the display (acoustic in the case of frogs and songbirds; visual in the case of lizards).

1.5 Introductory gestures (introductory notes) in different animals and their suggested function

1.5.1 Frogs: Introductory note provides conspecific information

Different frog species, like tree frogs and neotropical frogs, initiate their advertisement and aggressive calls with introductory notes. Two species of tree frog, namely *Hyla microcephala* and *Hyla ebracatta*, produce an introductory note with a longer duration as the distance between males of the species decreases. (Schwartz & Wells, 1985; Wells & Schwartz, 1984). Male *Hyla microcephala* increases the introductory note duration in response to the playback of higher-intensity aggressive calls (Schwartz & Wells, 1985). In leaf-folding frogs, where advertisement calls have a trill followed by a zip, female frogs respond more to a trill than a zip, while males respond to the zip component (Backwell, 1988). Similarly, *Geocrinia victoriana* advertisement calls consist of one introductory note followed by a repetition of notes; female frogs oriented toward the speaker playing back repeated notes rather than the speaker playing back introductory notes. Unlike the playback of repeated notes, males produced calls with longer introductory notes in response to the playback of complete calls and

introductory notes (Littlejohn & Harrison, 1985). Overall, this shows that the introductory note might be more suggestive of conspecific identity or male-male competition, while the female prefers other parts of calls.

1.5.2 Lizards: Introductory tail flicks and push-ups function as alerting component.

Lizards are known to perform introductory push-ups or introductory tail-flicks before their head bobbing display (visual display) (Fleishman, 1992; Peters & Evans, 2003). Introductory push-ups and tail flicks have been shown to function as an alerting component for the head bobbing display in different lizard species. Jacky lizards (*Amphibolurus muricatus*) increase the duration of introductory tail flicks in windy conditions and add more pauses (Peters et al., 2007). In order to characterize what properties of tail flicking evoke a maximum response from another lizard, Peters & Evans (2003) used a video display. They created an animation of a lizard tail using a video of an actual tail-flicking lizard. They showed that the response of an observing lizard to playback of both animation and original recording was the same. Following this, they modified the properties like duration, amplitude, etc of tail flicking. The response probability increases if tail flicking is of longer duration (Peters & Evans, 2003). A different species of lizard (*Anolis* lizard) also increases the amplitude of introductory push-ups when the receiver is further away (Fleishman, 1992). To test if introductory push-ups add an advantage to the sender in *Anolis* lizard, Ord and Stamps (2008) used a robotic lizard performing different displays. They used a species-typical head bobbing display with and without introductory push-ups and an artificial display (rapid dewlap extensions were used instead of introductory push-ups before the head bobbing display) under different light conditions. At a normal distance between the sender and receiver and under normal light conditions, they showed that the orientation time for the lizard was the same for all stimuli. However, during lower light conditions, orientation time was less for displays preceded by push-ups or rapid dewlap extension (Ord & Stamps, 2008). This suggests that the introductory push-up might function as an alerting component before the head bobbing display.

1.5.3 Songbirds: Introductory notes function as an alerting component, species recognition, and preparation for the upcoming song.

Oscine like zebra finch (Price, 1979; Sossinka & Böhner, 1980; Zann, 1996), java finch (Soma & Iwama, 2017), white crown sparrow (Soha & Marler, 2000), and blue-capped cordon blues

(Ota & Gahr, 2022), learn the song from a tutor and initiate songs with introductory notes. Suboscine (which don't learn song) like cocos flycatcher (Kroodsma et al., 1987), vermilion flycatcher (Chelén et al., 2005), and warbling antbird (Seddon & Tobias, 2006), also initiate songs with introductory notes. Even though different bird species initiate songs with introductory notes, the properties of introductory notes have not been compared across different bird species. This might be due to a lack of a clear definition of introductory notes. In most cases, vocalizations that are not part of the main message but rather occur in the beginning are considered an introductory note. Further, introductory notes have been suggested to perform a different function for various songbird species. The rufous-sided towhee song consists of an introductory note followed by trills. In his paper, Douglas G. Richards (1981) suggests that these introductory notes act as an alerting component while the trill carries the main message. These introductory notes are widely spaced tonal components with intermediate frequency vocalization, lacking amplitude modulation. The concentration of energy in one frequency band and the absence of modulation make it less affected by environmental noise like reverberation than the trill component, which spans a larger frequency range. Thus, making it a good candidate for long-distance communication. If these introductory notes precede the trills, then expecting a song after a given time, birds will likely pay more attention to the song signal embedded in the noise. Thus, he speculated it functions as an alerting component for long-distance communication and provides less information about species specificity. He measured response to different playbacks to confirm whether towhees use introductory notes as an alerting component. Playback of normal trill elicited an equal response as the playback of a normal and degraded song (consisting of both introductory notes and trill) generated by adding reverberation and filtered using a 3 kHz bandpass. While playback of degraded trill elicits a lesser response, suggesting the presence of an introductory element (degraded or normal) before trill enhances response. This suggests that the introductory notes function as an alerting component for towhee (Richards, 1981) (Figure 1.2A). Another suggested function of introductory notes is species identification in white-crowned and golden-crowned sparrows (Hudson & Shizuka, 2017; Soha & Marler, 2000). Soha and Marler (2000) tested the use of the introductory element in species recognition by tutoring juvenile white-crowned sparrows with playback of white-crowned sparrow song without an introductory whistle, heterospecific song preceded by white crown sparrow introductory whistle, and complete heterospecific song. They reported that juvenile white-crowned sparrow even learned the heterospecific song when preceded by an introductory element of the white-crowned sparrow (Soha & Marler, 2000) (Figure 1.2B). Thus, juvenile white crown sparrows learn songs preceded by a species-specific

introductory whistle, even when it is a heterospecific song. Predisposition for a species-specific introductory element can be inferred from the preference of the golden-crowned sparrow for its species-introductory element (Hudson & Shizuka, 2017). Overall, it suggests that introductory notes for both towhee and white-crowned sparrows are involved in communication. Apart from this introductory note (INs) have been suggested to function as a preparatory element for the song/motif in zebra finches. In zebra finches, INs are repeating short-duration vocalizations produced before the song. The number of INs produced by birds is variable across trials. The interval between successive introductory notes and the interval variability reduces from the first IN to the last IN (immediately before the first song syllable). Also, the INs structurally become more similar to the last IN and reach a consistent endpoint before the song (Rajan & Doupe, 2013). This progression of INs has been suggested to be similar to the motor preparation before the movement onset. The premotor and motor cortex show movement-related changes in neural activity for cued movements with a delay between the target appearance and the movement onset (Tanji & Evarts, 1976). Further, disruption of this activity in the premotor cortex during the delay period delays the onset of the movement (Churchland & Shenoy, 2007). The across-trial variability of activity in the premotor cortex of monkeys reduces after the instruction cue (during the delay period also). If the delay period is short such that variability is high, then monkey takes more time to initiate a movement. Thus, suggesting that activity in the premotor cortex reaches a consistent state before movement onset (Churchland et al., 2006). Thus, similar to motor preparation, INs reach a consistent endpoint (Figure 1.2C).

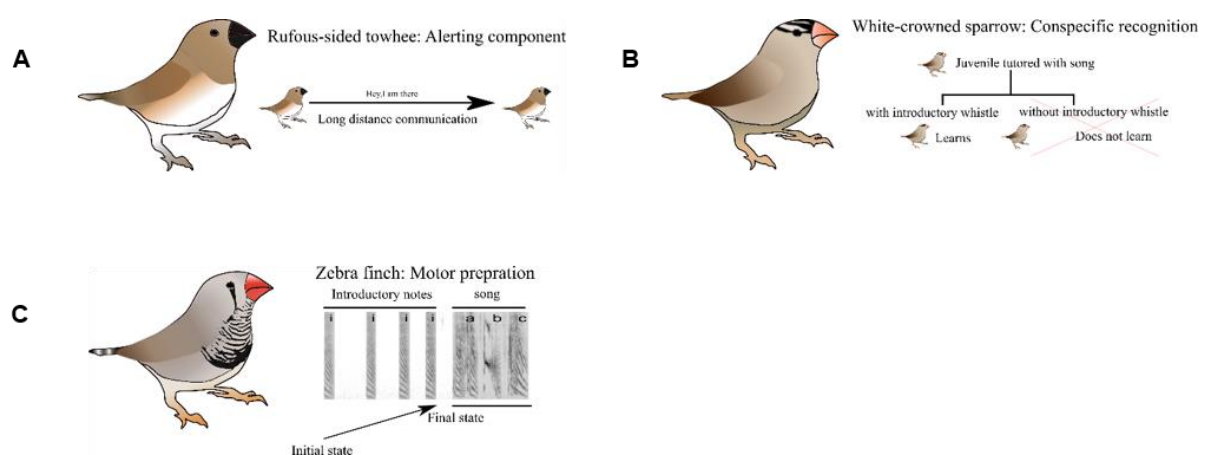


Figure 1.2 Different hypothesised functions for introductory notes in different songbird species.

A. Alerting component for rufous-sided towhee (Richards, 1981). B. Species identification for white crowned sparrow (Soha and Marler, 2000). C. Preparatory element for the upcoming song for zebra finches (Rajan and Doupe, 2013).

Overall, introductory gestures are movements that often occur at the beginning of a vocal or visual display. These could be a single note or a repetition of notes with variable spacing. The advertisement or aggressive calls produced by the frog start with a single introductory note. *Geocrinia Victoriana* advertisement calls start with an introductory note followed by a series of repeated notes (Littlejohn & Harrison, 1985). Other than this, lizards start their sequence of the species-specific display with a repetitive movement called an introductory tail flick or push-up (Fleishman, 1992; Peters & Evans, 2003). These always occur in the beginning and are more exaggerated compared to the main display. This makes it a good candidate for alerting the receiver in the noisy background. A species of lizard (*Jacky dragon*) initiates its display with a tail flick, which is followed by backward and forward arm waves and a push-up (Peters & Evans, 2003). In the case of songbirds, these are described as simple repeating vocalizations with variable spacing. Introductory notes in suboscine birds like warbling antbirds are repeating vocalizations that occur before an evenly spaced main phrase (Seddon & Tobias, 2006). Similarly, for oscine birds like java finches (Soma & Iwama, 2017), bengalese finches (Sakata et al., 2008), zebra finches (Price, 1979; Sossinka & Böhner, 1980; Zann, 1996), and blue-capped cordon-bleus (Ota & Gahr, 2022) introductory notes are a variable number of repeating vocalizations present at the beginning of a song bout (mostly the first vocalization, other than calls). However, in birds like white crown sparrows, the initial note is called the introductory whistle, which is followed by different phases (Harbison et al., 1999). There is no explicit/standard definition of introductory gesture across different animals, and this remains to be addressed.

Given all the different functions of the introductory notes above, we can say that INs are mostly involved in communication in noisy environments or species recognition. Apart from communicative function, in the case of the zebra finch, it has been suggested to function as a preparatory element for the upcoming song. Thus, different roles have been attributed to the introductory notes across different animals (Table 1.1). The presence of introductory notes before displays in many animals and different modalities makes it interesting to study. Animals, irrespective of learning communicative signals from the tutor, initiate displays with INs. Different functions have been suggested for INs in different animals, but all functions have not been tested in one species. In the case of vocal communication, INs are also vocalizations. Does this mean it can serve both communicative roles, providing species specificity and preparation for the upcoming song for birds? One possibility is that the property of INs influences its function. In all the cases where INs is a single note, it might function as an

altering/communicative function. However, for zebra finch, where INs repeat, it might have both communicative and preparative functions. Thus, to understand the function of INs, it is important to understand its properties. One unanswered question is the origin of these INs and its property, especially for the learned display. I have used zebra finch as a model system to study the origin of INs. Both communicative and preparatory speculations require birds to produce INs irrespective of exposure to the tutor. Given that INs are also vocalization, like songs, certain properties of INs could be influenced by learning from the tutor, thus resulting in nest-specificity. Most studies have focused on learned/innate properties of the song but rarely looked at introductory notes from the perspective of learning from the father, like a song. Thus, the origin of INs needs to be addressed.

Table1.1. Hypothesized function and properties of introductory notes

Animal	Call/song type	Hypothesized Function	Note type
Frogs	Produced before advertisement/aggressive call	Male-male competition/ conspecific identification	Single
Lizards	Produced before head bobbing display	Alerting component	Repetitive
Birds			
Towhee		Alerting component for long-distance communication	Single
White crown sparrow	Initiate the song with introductory notes/note	Species specificity for song learning	Single
Zebra finch		Preparation for the upcoming song	Repetitive

1.6 Zebra finch: a good model system to study introductory notes

The Zebra finch (*Taeniopygia guttata*) is an oscine native to Australia. Only the male zebra finch produces a song as a part of its courtship ritual. Zebra finches begin their song with repetition of a variable number of short-duration vocalizations called introductory notes (INs),

followed by a consistent sequence of vocalizations called a motif/song. Songs are produced both in the presence (directed song) and absence of females (alone song) (Price, 1979; Sossinka & Böhner, 1980; Zann, 1996). Zebra finch learns song from its tutor, which is usually its father. Zebra finch song does not change in adulthood, and it can produce the same song with great consistency, making it a good model system for studying learned motor movement. Further, the presence of both directed and undirected songs makes it a good model system for studying communication. It allows the comparison of song properties in the two different contexts. This difference in properties of the song in an alone context (in the absence of a female) relative to directed might hint at its function in communicating with the female. Zebra finches reared without exposure to the song also produce songs with species-specific song patterns, i.e., INs and motifs (Price, 1979). This makes the zebra finch a good model system to discern the relative contribution of learning and innate components. To understand the communicative or preparatory function of introductory notes, it is important to separate properties that the juvenile learns from the father and ones that it acquires, irrespective of exposure to the father.

1.7 Song learning in zebra finches

Male zebra finches learn the song mainly from their father or available tutor (Zann, 1990). They learn song during the early phase of life and do not change following crystallization in adulthood. Male zebra finches reared with their father till post-hatch day 35 produce songs similar to their father's song. This similarity matched those reared with father till post-hatch day 100 (Böhner, 1990). This suggests that initial exposure to the tutor's song until day 35 is sufficient for learning the song. Song learning has two phases: the sensory acquisition phase and the sensory-motor phase (Immelmann Klaus, 1969; Marler, 1990) (Figure 1.3). During the sensory acquisition phase, juvenile zebra finches memorize the song produced by their father.

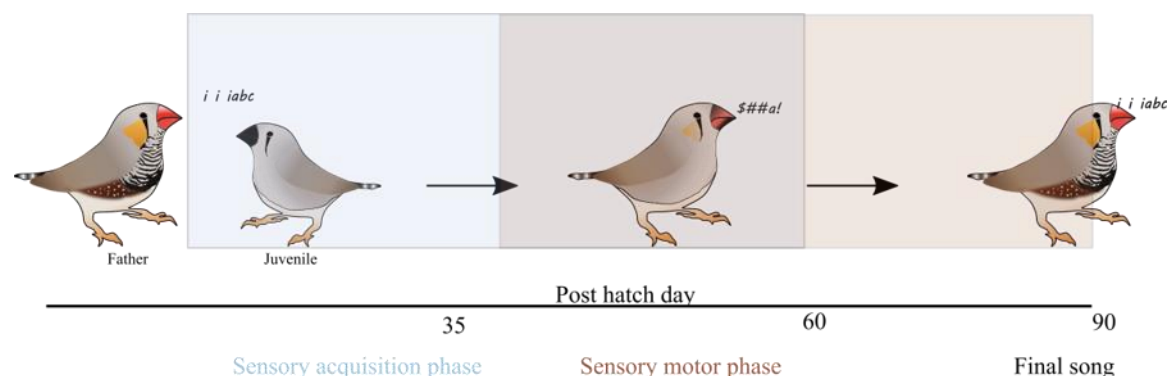


Figure 1.3 Schematic of stages of song learning for the male zebra finch

Blue marks the sensory acquisition phase, during which juvenile (black beak) learns father's song (red beak, left most bird). Song is represented as 'iiiabc'. Brown marks the sensory motor phase, during which juvenile tries to produce vocalization similar to father (middle birds, red-black beak). '\$##a!' represent subsong.

This phase lasts till post-hatch day 60. The sensory-motor phase lasts from post-hatch day 35 to post-hatch day 90, where juveniles practice their song and produce vocalizations similar to the memorized template.

During the song-learning phase, male juvenile zebra finch transits between three types of song (Immelmann Klaus, 1969; Marler, 1990). Initially, it produces subsong, which is a series of unrecognizable vocalizations. Subsong is considered similar to babbling in humans (Marler, 1970). Following subsong, it produces a plastic song. The plastic song consists of a few recognizable syllables, but the syllables' order is inconsistent across trials. After the plastic song, it produces the final crystallized song (Figure 1.4). The syllables and sequence of syllables do not change after this. Other than changes in the structure and sequence of syllables, the interval between syllables also changes. The gaps between syllables reduce in duration, and the variability of interval also reduces (Glaze & Troyer, 2013). Even though zebra finch learns song, few properties of the song have species-specific characteristics; irrespective of exposure to the tutor's song during the song-learning phase, birds will produce a song consisting of some innate features (Price, 1979). In the following section, I will summarize the innate features of the male zebra finch song and what is known about introductory notes.

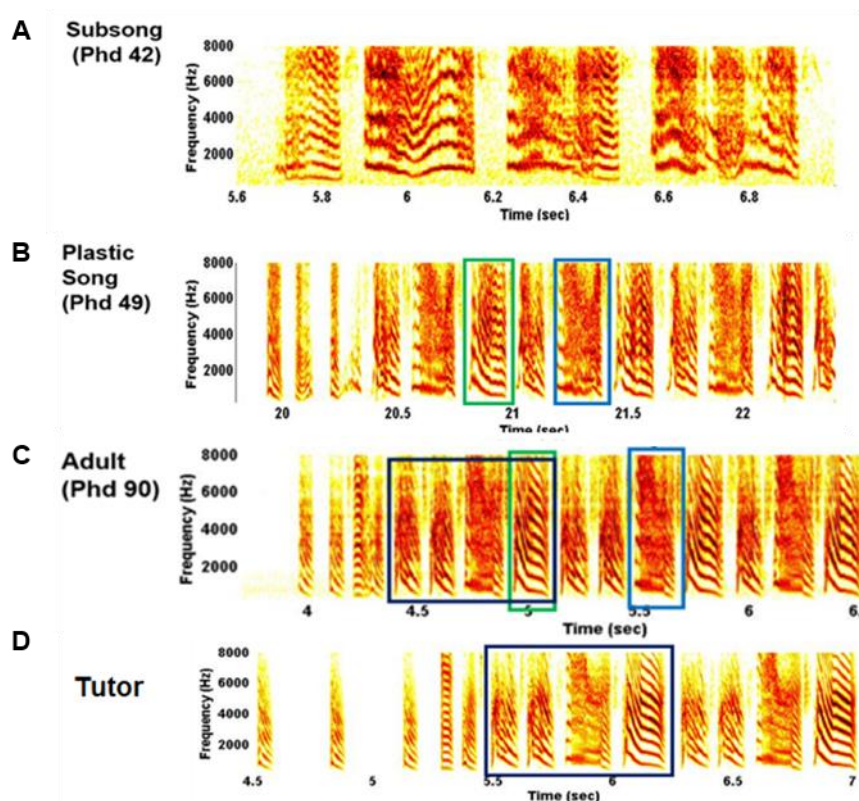


Figure1.4 Spectrogram of song produced by male zebra finch during the song learning phase

A. Subsong produced by bird around Phd 42 (Post hatch day). It consists of unrecognizable syllables. B. Spectrogram of plastic song produced by the same around Phd 49. Green and blue boxes highlight recognizable syllables found at day 90 as well. C. Spectrogram of song produced by same birds at day 90. D. Spectrogram of the tutor's song. Dark blue rectangle represents motif that is similar between tutor and pupil.

1.7.1 Innate component of the male zebra finch song

- Male juvenile zebra finches have an innate preference for the zebra finch song. Thus, when given an opportunity to listen but not bond with a conspecific and heterospecific tutor, it prefers to learn song produced by a conspecific (Immelmann Klaus, 1969).
- Isolate zebra finches that are birds reared in the absence of any adult male zebra finch produce song consisting of introductory notes followed by the motif. Birds that are deafened early during the song-learning phase or later in adulthood initiate the song with introductory notes (Price, 1979). Further, birds tutored by electrical stimulation of a specific brain region involved in song learning also initiate songs with introductory notes (Zhao et al., 2019). This suggests that zebra finches have a biological predisposition to initiate songs with introductory notes.
- As mentioned before, zebra finches produce both directed and undirected songs. Directed song has more introductory notes and a faster tempo and is less variable than undirected (Sossinka and Böhner, 1980). Logan et al. showed that birds tutored by playback without a tutor or female also show a similar difference between the directed and undirected song (James et al., 2018). This suggests the tendency to change songs is innate and not learned from the father.
- Zebra finches prefer the temporal pattern of syllables similar to normal zebra finch songs. When juvenile zebra finches tutored with bengalese finch songs, learn syllables produced by the bengalese finch but string them together in the temporal pattern of the zebra finch song. In the same study they have shown that Field L, one of the auditory regions in the zebra finch's brain, responds to strings of syllables or noise separated by silent gaps similar to that found in the zebra finch song (Araki et al., 2016). This suggests they have an innate preference for the temporal pattern of the zebra finch song.

In the following section, I will summarize what we know about introductory notes produced by zebra finches.

1.7.2 What do we know about the properties of introductory notes

- Zebra finches begin their song with a variable number of introductory notes. The introductory note duration is shorter than motif syllables (Rajan & Doupe, 2013).
- The interval between successive INs reduces (temporal progression) as it approaches the motif, and the introductory notes become more similar to the last INs (acoustic progression). Also, the variability of the interval reduces. Thus, similar to motor preparation, where across trial variability in the premotor cortex reduces and reaches a consistent point before the start of a movement, introductory notes reach a consistent endpoint before the start of the motif (Rajan & Doupe, 2013).
- The previous vocalization influences the number of introductory notes produced by birds. The number of introductory notes birds produce reduces if a call precedes the IN by less than 200 msec. Further, removing auditory and proprioceptive feedback doesn't affect IN progression or the number of INs. This suggests that sensory inputs to the brain does not influence these properties of IN, rather it is controlled within the brain (Rao et al., 2019).

1.8 What do we know about brain regions involved in song production and learning

Different brain regions are important for song production and learning. These form part of the song system pathway. Historically song systems in zebra finches have been divided into the motor pathway (required throughout life for normal song production) (Nottebohm et al., 1976) and the anterior forebrain pathway (required only for song learning in juvenile zebra finches and song plasticity in adult zebra finches) (Bottjer et al., 1984; Brainard & Doupe, 2000; Scharff & Nottebohm, 1991; Williams & Mehta, 1999). HVC (proper name) is one of the premotor brain regions responsible for producing stereotyped songs by adult zebra finches. HVC lesion in adult male zebra finch results in loss of both acoustic and temporal pattern of the song (Nottebohm et al., 1976). However, similar lesions in juvenile male zebra finch during the subsong stage has no effect (Aronov et al., 2008). HVC projects to a motor region called RA (robust nucleus of the arcopallium) (Nottebohm et al., 1976). The lesion in RA prevents song production, but bird can produce calls. RA controls the acoustic features of the song; it projects to nXIIIts (a tracheosyringeal portion of the hypoglossal nucleus) which in turn projects to the syringeal muscles. RA also projects to respiratory centers in the brain stem like RAm (nucleus retroambigualis), PAm (nucleus paraambigualis), and RVL (rostral ventrolateral medulla)(Wild, 1993). These brain regions form the song motor pathway (Nottebohm et al.,

1976; Simpson & Vicario, 1990). Thus, the motor pathway controls respiratory and vocal motor neurons to produce song. On the other hand, the anterior forebrain pathway, which is for song learning and plasticity, consists of basal ganglia nucleus AreaX, thalamus region called DLM (dorsolateral nucleus of the medial thalamus), and a critical region called LMAN (lateral portion of the magnocellular nucleus of the anterior nidopallium). HVC, apart from sending projections to RA, also projects to AreaX. AreaX projects to DLM, which in turns project to LMAN, and LMAN projects to RA (Bottjer et al., 1989) and projects back to AreaX (Vates & Nottebohm, 1995). This forms the cortical basal-ganglia cortical loop similar to mammals (reviewed in Fee & Scharff, 2010). AreaX lesions in juvenile zebra finches increase variability (Sohrabji et al., 1990), whereas lesions in LMAN result in early maturation of the song and affect similarity to the tutor's song. In contrast, similar lesions in adult zebra finch do not affect song (Bottjer et al., 1984; Scharff & Nottebohm, 1991). DLM lesions in juvenile birds produce an effect similar to LMAN lesions, increasing stereotypy (Goldberg & Fee, 2011). As mentioned before, HVC lesions in adult male zebra finches leads to the production of subsong. Following LMAN lesion, this anterior forebrain pathway driven subsong is abolished, in birds which have already received HVC lesion. Further, DLM lesions following HVC lesions or vice versa also reduce the amount of singing. This suggests DLM, which provides input to LMAN, might be responsible for initiating anterior forebrain pathway-driven subsong (Chen et al., 2014). Apart from these two pathways, a few other regions are involved in song production and learning. NIf (nucleus interfascialis of the nidopallium), an auditory-motor region, also affects song learning. NIf forms a bidirectional connection with the auditory region called avalanche and also projects to HVC. It also receives input from uvaformis (Uva), a thalamic nucleus (Akutagawa & Konishi, 2010; Nottebohm et al., 1982). NIf lesions in adults does not affect the song. However, its lesion in juveniles affects song learning. Birds with NIf lesion before exposure to the tutor don't copy the tutor's song, while birds that receive NIf lesion after tutoring produce an accurate copy of the tutor's song (Roberts et al., 2012; Zhao et al., 2019) (Figure 1.5). Uva also projects to HVC. Uva receives inputs from respiratory nucleus called nucleus retroambiguus (PAm) and mid brain nucleus, the dorsomedial nucleus of the intercollicular complex (DM) (Ashmore et al., 2008; Reinke & Wild, 1998; Striedter & Vu, 1998). In adult zebra finches, following bilateral lesion in Uva, the undirected song becomes like a subsong and for directed song, birds produce a string of IN-like vocalizations without characteristic temporal progression (Coleman & Vu, 2005; Danish et al., 2017; Williams & Vicario, 1993).

The song system also receives neuromodulatory inputs from regions like locus coeruleus (LC) (Singh Alvarado et al., 2023), ventral tegmental area (VTA), substantia nigra pars compacta (SNc) (Gale et al., 2008; reviewed in Kubikova & Košťál, 2010; Lewis et al., 1981; Reiner et al., 2004) and A11 (Ben-Tov et al., 2023). LC provides non-adrenergic inputs to the song system. Katic et al. (2022) have shown that the activity of LC neurons increases during the playback of a tutor's song in juvenile zebra finch (Katic et al., 2022). Further, inhibition of NCM (caudomedial nidopallium involved in storing the tutor's song memory) (Gobes & Bolhuis, 2007) projecting LC neurons during the exposure to the tutor's song affects the accuracy of song copying by the juvenile zebra finch (Katic et al., 2022). VTA and SNc provide dopaminergic inputs. These have been suggested to evaluate song performance in adult zebra finches (Duffy et al., 2022; Gadagkar et al., 2016). Overall, neuromodulatory inputs to the song system affect song learning and production.

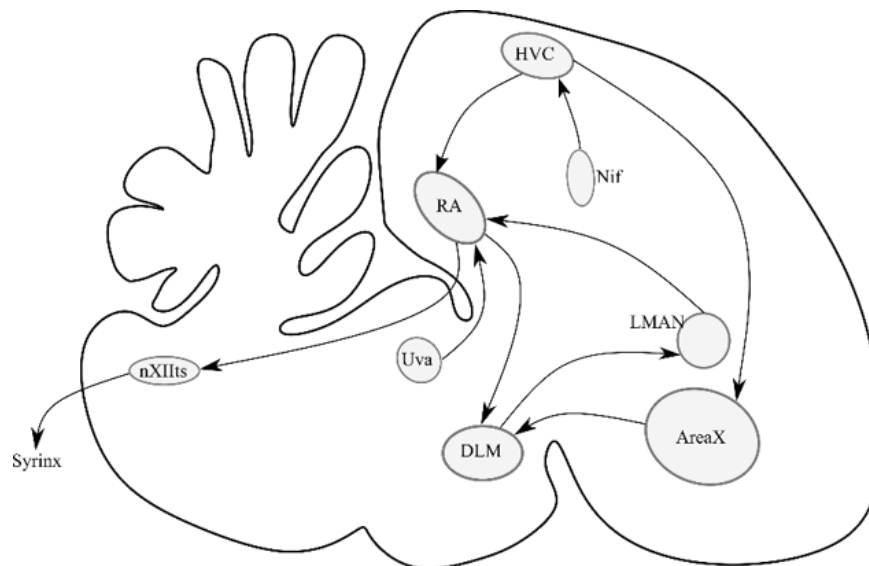


Figure 1.5 Sagittal section of male zebra finch brain highlighting different brain regions involved in song learning and production.

HVC (proper name), RA (robust nucleus of the arcopallium), DLM (dorsolateral nucleus of the medial thalamus), LMAN (the lateral portion of the magnocellular nucleus of the anterior nidopallium), Nif (nucleus interfascicularis of the nidopallium), Uva (Uvaeformis), nXIIIts (tracheosyringeal portion of the hypoglossal nucleus).

1.8.1 What we known about brain regions involved in IN production

- HVC controls the timing of syllable sequence in a song in zebra finch. Cooling of HVC increased the duration of both syllables and intervals between the song. Apart from the syllables, the duration of introductory notes also increased. However,

introductory notes stretched lesser compared to song syllables upon cooling. This suggests that the brain regions affecting the production of learned features of the song have some influence on the production of introductory notes (Long & Fee, 2008).

- Rajan & Doupe (2013) showed that the HVC interneuron activity across trials is highly correlated for the last introductory note (IN just before the first motif syllable) and different from the preceding INs. For another class of neuron, namely AreaX projecting HVC neurons, for some neurons firing rate was different for the last position, suggesting a unique representation of the last IN (IN immediately before the first motif syllable) (Rajan & Doupe, 2013).
- As mentioned before, juvenile zebra finch learns songs from its tutor, and one of the brain regions that influence song learning is NIf. Juvenile zebra finches fail to learn the tutor's song following NIf lesion before exposure to the tutor. However, similar lesion after tutoring does not affect learning (Roberts et al., 2012; Zhao et al., 2019). NIf projects to HVC. Following optogenetic activation of these projection neurons during tutoring, the juvenile birds eventually produce adult songs consisting of syllables of similar duration as the duration of optogenetic stimulation. Interestingly, these birds also initiated songs with the repetition of introductory notes (Zhao et al., 2019). This suggests birds have an innate tendency to initiate songs with introductory notes. NIf may or may not be involved in coding the number of times birds repeat introductory notes.
- Another brain region involved in the production of song sequences is Uva; following lesion in Uva in adult birds, birds produce subsong-like vocalizations. In the directed song, birds with uva lesions produce strings of introductory note-like vocalizations without characteristic temporal patterning, which doesn't progress to the song (Coleman & Vu, 2005; Danish et al., 2017; Williams & Vicario, 1993). This suggests similar to HVC; Uva is involved in controlling the properties of the song. A recent study has shown that following disruption of Uva activity during singing, birds stop singing only at the end of a syllable. This suggests Uva might provide input about the start of syllables to HVC. Further, Uva fires at the onset of introductory notes (Moll et al., 2023).
- Ts nerve provides motor input to the syrinx (vocal organ). Damage to the Ts nerve affects efferent and afferent motor commands to/from the syrinx (Bottjer & Arnold, 1982, 1984). Damage to Ts nerve in adult zebra finch, which results in loss of motor input and proprioceptive feedback from the syrinx, does not influence the number and

temporal progression in INs. Similarly, removing auditory feedback does not affect the number and temporal progression of introductory notes. This suggests birds do not require real-time auditory or proprioceptive feedback for evaluating the repetition of INs (Rao et al., 2019).

1.9 Objectives

Zebra finches reared in the absence of a tutor also initiate song with INs (Price, 1979; Sossinka & Böhner, 1980; Zann, 1996). This suggests zebra finch has an innate tendency to initiate song with INs also providing species-specific characteristics. Further, brain regions that affect song production also seem to affect the production of INs. But, unlike song, INs are variable in number across trials and have variable intervals. Given that we know that the song has both learned and innate properties, little is known about the origin and properties of INs, especially in the context of learned vocalization. As mentioned before, different functions have been suggested for INs in different songbird species. Understanding its origin will shed some light on its function. In this thesis, I have looked at the following aims regarding the origin and function of introductory notes in zebra finch.

Aim1. Understanding the origin of introductory notes

The first step towards understanding the origin of introductory notes is to check if they also have a learned component, like the song. Here, I have looked if juvenile zebra finches learn introductory notes from the tutor in the same way as the song. I have used different tutoring methods to answer this question.

Aim2. Characterize innate/learned properties of introductory notes

Given that zebra finch reared in a tutor's absence, initiate song with introductory notes. Next, I asked if the properties of INs produced by these are the same as birds reared in the presence of a tutor. Any common properties would support the function of INs in preparation for the upcoming song, for I have compared the properties of introductory across different groups of birds. Namely, birds reared in the absence of exposure to introductory notes and the presence of a tutor.

Aim3. Brain region involved in the learning of introductory notes

If learning of INs is similar to acquisition of song, one would expect brain regions involved in song learning to influence the learning of INs as well. As mentioned before, LMAN is one of the brain regions that affect song learning in zebra finches (Bottjer & Arnold, 1984; Scharff & Nottebohm, 1991). Here, I have looked at its role in learning introductory notes in the male zebra finch.

Aim 4. Understanding the function of introductory notes

Male zebra finches sing both in the presence (directed) and absence (alone) of female. Female-directed song has higher number of INs preceding it than alone song (Sossinka & Böhner, 1980). One reason is the female might prefer a song with more INs. Here, I have looked at the preference of female zebra finches for song starting with INs. In addition, zebra finches also produce song starting with higher number of INs when singing to a juvenile (Y. Chen et al., 2016). Thus, INs could influence juvenile's attention towards tutor's song and ultimately affect accuracy of song copying. To test if it is true in zebra finches, I have looked at the influence of the number of INs produced by the father on the accuracy of song copying by juvenile zebra finches.

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Chapter 2

Understanding the origin of introductory notes

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Introductory gestures before songbird vocal displays are shaped by learning and biological predispositions

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2.1 Introduction

Animals produce various complex displays to communicate with their conspecifics [1]. Many of these communicative displays begin with the repetition of introductory gestures. For example, *Anolis* lizards produce a few introductory “push-ups” or “tail-flicks” before starting their head bobbing display [2,3], frogs and toadfish produce introductory vocalizations before their advertisement calls [4,5] and many songbirds also produce introductory vocalizations before the start of their complex songs [6–17]. The functions of these introductory gestures remain unclear, but multiple hypotheses exist based on independent observations in different animals. All of these functions relate to the main vocal display and can broadly be divided into functions that enhance the communicative aspect of the display or motor preparatory functions. For instance, under noisy conditions, the main part of the display becomes more difficult to detect and theory predicts the addition of a “simpler” signal immediately before the display to ensure reliable detection [18]. This hypothesis is supported by experiments in songbirds and *Anolis* lizards. Territorial songbirds typically respond to playbacks of song from a speaker. Rufous-sided towhees respond less to degraded versions of song, but the addition of simpler introductory vocalizations before degraded song increases the magnitude of the response [19]. Similarly, *Anolis* lizards respond to visual head-bobbing displays of other lizards and under low light conditions, respond more strongly to displays that begin with introductory push-ups [20].

While these studies suggest that introductory gestures add value to the communicative aspect of the display by enhancing detectability, other studies suggest that introductory gestures add value by providing recognition signals. For example, naive, young songbirds learn their songs from adults of their species and an important problem for young birds is choosing the right songs to learn. Introductory vocalizations appear to help in this selection; white-crowned sparrows can reliably learn songs of their species and even songs of other species from tape playbacks, only when these songs are preceded by the characteristic introductory whistle of white-crowned sparrows [21]. Further, this characteristic introductory whistle differs subtly between different geographical populations and birds of a particular region respond more strongly to playbacks that begin with region-specific introductory whistles, even if the songs that follow are from a different region [22]. Thus, introductory gestures could enhance the main

display by increasing detectability (“alerting” signals) or by providing signals for recognition (“species” / “local-dialect” identity).

More recent studies suggest a motor preparatory function for introductory gestures [23,24]. Motor preparation is believed to represent a brief period prior to movement, when the brain “prepares” parameters of the upcoming movement [25]. The idea of motor preparation comes from the observation that movements towards a pre-specified target are faster when a delay period is introduced between target onset (representing the instruction) and the “GO” cue; longer the delay, faster the onset of movement after the “GO” cue suggesting “more” prepared responses following longer delays [25,26]. Neural recordings in primates and rodents have shown the presence of “preparatory” neural activity during this delay period and disruption of this preparatory activity delays movement onset [25,27]. This preparatory activity is believed to drive neural activity from a variable “initial” state, at the time of target onset, to a more stereotyped, “final” state from which movements can be readily generated [25]. Our recent experiments with the zebra finch, a well-studied Australian songbird [14], are consistent with a role for introductory gestures in motor preparation [23,24]. Specifically, zebra finches, generally precede their songs with a series of stereotyped introductory notes (INs), which are relatively short in duration and simple in spectral content (see Fig. 2.3A for an example of zebra finch INs) [8,10,14,28]. INs progress from a variable first IN to a more stereotyped last IN, just before the start of each song [23,24], similar to the progression of neural preparatory activity described in primates [25].

While the function of INs is unclear, recent studies in songbirds have also shown considerable variation in the number of IN repeats and IN acoustic structure between individuals of a given species [24,28–31]. (Fig. 2.3B; example of variation seen in zebra finches). What is the source of this variation? Variation in elements of song between individuals is a consequence of learning [8,10,32,33] and similarly, variation in IN number and IN acoustic structure could reflect variation in learning from different tutors. Another possibility is predicted by the motor preparation hypothesis. Motor preparatory activity in primates, is different for different upcoming movements [25]. By analogy, if INs helped prepare the songbird brain for song production, one would expect different number/structure of INs for different songs that follow. This would predict a correlation across birds between variation in IN number and/or structure and variation in the song that follows. Finally, variation in IN number and structure across birds could also be a result of biological predispositions similar to those involved in the production of elements of song [34,35]. Here, using natural and experimental tutoring methods, I examine

these different predictions in the zebra finch. My results demonstrate that, similar to song elements, INs are shaped by both learning and biological predispositions and suggest mechanisms for generating variation between individuals, while still maintaining the species-specific structure of complex displays, like birdsong.

2.2 Materials and Methods

All procedures at IISER Pune were conducted after approval from the Institute Animal Ethics Committee (IAEC), IISER Pune and were in accordance with guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), New Delhi. All procedures at McGill were conducted following approval from the McGill University Animal Care and Use Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Zebra finches obtained from an outside vendor or bred in IISER Pune or McGill were used for all experiments. Detailed methods are present in Supplemental Information.

2.2.1 Song recordings

All birds were recorded when they were adults (> 85 days post-hatch) and only undirected songs in the absence of any other bird were recorded. I chose to examine undirected songs because they provide a more controlled condition for quantifying IN number and structure. For example, INs before directed songs are influenced by distance from the female [36] and quality of the stimulus [37]. In addition, the initial, softer INs can be lost in the increased background noise, typically associated with directed songs because of movement of both birds and female vocalizations. Songs were recorded in custom-made sound attenuation boxes (NewTech Engineering Systems, Bangalore, India; TRA Acoustics, Ontario, Canada) by placing an omnidirectional microphone (AKG 517; Countryman Associates, Menlo Park, CA) on top of the bird's cage. Signals from the microphone were band-pass filtered, digitized and recorded to a computer ($n=132$ birds). Songs were recorded on a computer using either a sound card (44.1 kHz for 128 birds) or a data acquisition board (32kHz for 4 birds; National Instruments, NIDAQ PCI). Sound was either recorded continuously for extended periods of time (continuous recordings) or small chunks of data were recorded when the signal crossed a pre-set threshold (triggered recordings). In triggered recordings, 2-3 seconds of data before and

after threshold crossing were saved along with the data that crossed threshold. All birds were recorded when they were adults (> 85 days post-hatch) and only undirected songs in the absence of any other bird were recorded. For most analyses (n=127/132 birds), only files with > 2s of silence before and after song bouts in a file were considered [28]. For the other 5 birds, I considered files with > 1.5s (4 birds - > 1.9s; 1 bird - > 1.5s) as I did not have enough files with 2s silence

2.2.2 Experimental groups

I used 4 different experimental groups in this study.

2.2.2.1 Normally reared birds

A total of 16 nests were analyzed with 13 nests having at least 3 offspring (range: 1-9). Juveniles were housed with their parents and siblings until they were 50-94 days old, after which they were transferred to the colony and housed with other males from same or different families. All birds used in this group (n = 65, nests =16) were bred at IISER, Pune. Twelve of the fathers were purchased from outside. The four lab-bred birds used as fathers were not included as offspring for their nests to avoid data duplication. For two of the nests, two different females were sequentially used for breeding, and offspring from both females were included as part of the nest. For a small number of birds (5/81), I combined data across multiple days to collect > 35 bouts for analysis (n=3 birds, 2 consecutive days combined; n=1 bird, 3 days, spread across a period of 10 days, combined; n=1 bird, 4 days, spread across a period of 6 days).

2.2.2.2 Isolation procedures for all other groups

For all other groups outlined below, I used naive birds (i.e. birds that had not previously learned song) to test various hypotheses about IN learning. In order to prevent song learning from their father, juvenile zebra finches were separated from their father around phd 10 (range phd 6-16) and reared with their mother and siblings until phd 35 (range: phd 34-54). Following this, juveniles were separated and housed individually in small cages (except for 2 birds who were housed together). These birds were then used for the different groups as outlined below. Previous studies have shown that exposure to the father's song before phd 25 does not significantly impact learning [47], so these birds are unlikely to have learned their father's song during this period.

2.2.2.3 Socially reared birds

14 birds were tutored with an adult male different from their father using a social rearing paradigm used in earlier studies [38,39] (Fig. 2A; $n=10$ at IISER Pune and $n=4$ at McGill; total $n=14$ birds from 11 different nests). For tutoring, a cage with an adult male (tutor) was placed next to the juvenile's cage. For the 10 birds tutored at IISER Pune, tutors were chosen after considering their mean IN number and that of the father. The absolute difference in mean IN number between father and normally reared son was 1.08 (95% CI is 0.89-1.31). To better reveal the strength of IN learning, I chose social tutors with mean IN number > 1.32 ; i.e. outside the 95% CI for difference between father and normally reared son. (mean difference in IN number between father and tutor – 2.75; range: 1.37 – 3.48). Birds tutored at McGill were part of a different study where song (but not IN) learning was described [39]. For these birds, INs of the tutors was not a consideration during the choice of tutor. Therefore, to investigate IN learning, only a subset of these tutored birds were analyzed here. Specifically, only socially tutored birds, where recordings of both father and tutor were available and the mean difference in IN number between these two was > 0.5 (mean difference in IN number between father and tutor – 2.06; range: 0.5-4.78), were included in the analyses. In fact, I observed a negative correlation between mean IN number of socially reared birds and mean IN number of their fathers (Figure 2.5D) reflecting the choice of social tutors with IN number different from their fathers.

The tutoring phase lasted ~1.5 months (phd 34-40 to phd 91-97) for birds tutored at IISER Pune and for 5 days for birds tutored at McGill. Significant song learning is observed for socially reared birds even after just 5 days of tutoring [39].

2.2.2.4 Playback tutored birds

Using active tutoring methods [38,40], birds were tutored with synthesized zebra finch songs, without INs, that were played back through a speaker (Figure 2.12A; $n=22$ birds from 10 different nests) at McGill ($n=13$) or at IISER Pune ($n=9$).

Tutoring procedures varied slightly across locations. McGill birds were operantly tutored using perch hops. Briefly, each time juveniles hopped on a perch, they were presented with one bout of song from a speaker placed outside the cage. Each day was divided into 3 periods and birds were presented a maximum of 10 song bouts during each period. After the maximum number

had been reached during a period, further perch hops did not elicit song playback. Each bout consisted of 4 motifs consisting of the same 5 distinct syllables. Importantly, repetitive INs were not part of the song playback.

Birds at IISER Pune were tutored using a similar paradigm. Briefly, a dummy bird was placed in the cage with the juvenile and each time the juvenile pecked the dummy, songs were played back through a speaker placed outside the cage behind the dummy. For six of the birds, each peck elicited the playback of a song bout consisting of two motifs without repetitive INs (see Figure 2.12A for example). For three of the birds, each peck elicited either a long call or a song bout with two motifs without repetitive INs. Birds were tutored for an hour in the morning and an hour in the evening. There was no limit on the total number of songs or calls played back. If there was a long period (~10-15 minutes) during which the juvenile did not peck the dummy, one song bout was played back passively. For 3 of the birds, stimuli were also passively played back at the end of the session (10 song bouts and 10 bouts of long calls). Tutoring lasted 1-2 months (phd 25-30 to phd 60-93). Outside of the tutoring sessions, playback-tutored birds were kept in visual but not acoustic isolation from other birds. Two birds were tutored together in a cage with a dummy bird. For 7 birds, the motif of its father was used for playback and each playback bout consisted of two of these motifs without INs. For 1 bird, I played back the motif of a bird different from the father. For 1 bird, the motif played back consisted of one IN of the father and the father's motif consisting of 2 syllables. Importantly, for this bird also, there were no repeating syllables (INs) before the motif.

2.2.2.5 Isolate birds

5 lab bred male zebra finches (from two different nests) were reared in isolation. After birds were separated from their mother and siblings, they were kept in visual but not acoustic isolation from other birds. Earlier studies have shown that visual isolation is sufficient to prevent song learning from other birds [41].

2.2.3 Data analysis

All analyses were conducted using custom written scripts in Matlab (Mathworks). Scripts to generate figures used panel [42].

2.2.3.1 Song segmentation and syllable categorization

Song files were segmented and labeled as described previously [23,24]. Zebra finch songs are produced in bouts that consist of multiple renditions of a motif (a stereotyped sequence of syllables). Song files were first segmented using an amplitude threshold with a minimum syllable duration of 10 ms and minimum inter-syllable gap of 5ms. Syllables were labeled with a modified template matching algorithm [55] and manually checked. Groups of syllables with silence > 2s before and after the group were considered as “bouts”. Gaps of 2s or longer between syllables has been shown to mark the boundaries between bouts [28]. To see whether this particular gap to identify bouts affected the conclusions, I used gaps between 1.5s and 2s as criteria to identify bouts. For each of these intervals, the strength of the correlation in mean IN number between father and son was similar and remained significant for all intervals (1.9s: $r = 0.6$, $p = 1.131 \times 10^{-7}$; 1.8s, $r = 0.6$, $p = 1.214 \times 10^{-7}$; 1.7s, $r = 0.599$, $p = 1.326 \times 10^{-7}$; 1.6s, $r = 0.594$, $p = 1.778 \times 10^{-7}$; 1.5s, $r = 0.592$, $p = 2.063 \times 10^{-7}$).

For every bird, a motif was first defined as a fixed sequence of syllables that appeared consistently in a majority of song bouts. Syllables that occurred in isolation or occurred at random positions in song bouts were classified as calls. Syllable types that were repeated a variable number of times before the first motif of a song bout were considered INs. 68.18% (90/132) birds had one IN type, 23.48% (31/132) had two types of INs, 5.3% (7/132) had more than 2 types of INs and 3% (4/132) had no INs. Figure A1 shows the motif syllables, INs and calls for one example bout for all of the normally reared birds from 16 nests classified as per the above scheme.

2.2.3.2 IN number calculation and associated controls

To count IN number for each bout, I started with the introductory note just before the first motif and counted backwards until either the inter-syllable interval was > 500 ms or the syllable was not an IN [23,24,43] (see Figure A1). For birds with multiple types of INs, all types of INs were included in the calculation of IN number.

There was variation in the sample size of birds in each nest. To check whether this difference in sample size could influence the correlation in IN number between fathers and normally reared birds, I used a random reassignment procedure to assess significance of the observed correlation. For this procedure, all sons were randomly reassigned to different fathers, while

maintaining the actual number of birds per nest, and then the correlation between fathers and sons was calculated. This procedure was repeated 10000 times and the 95% confidence intervals were estimated. This same randomization procedure was also used to assess the significance of the correlation between socially-tutored birds and their tutors. In this case, the total number of socially tutored birds was maintained constant during the random reassignment procedure.

While classifying syllables as INs or motif syllables, I was not blind to the relationship between birds or the experimental group. To control for any potential biases that this might have introduced in my classification of INs and motif syllables, I also used a script based categorization of INs and motif syllables. All of my comparisons of mean IN number between father and son, tutor and pupil, etc. for all groups of birds were verified using this script-based classification. All of the results using the script-based classification agreed with the results using the manual classification.

2.2.3.3 Script based classification of syllables

To decide on criteria for classifying syllables as INs or motif syllables, I used song recordings from a set of 20 birds that were not part of this study. These comprised song recordings from birds that were bought from outside vendors or birds that were used in previous studies [23, 24, 36]. In these birds, I identified INs (n=25), motif syllables (n=81) and calls (n=51) as described above for all syllables that occurred as part of song bouts. For each syllable type in each of the birds, I calculated the self-repetition probability and the probability of the syllable occurring at the beginning of the bout. Most syllables that I categorized as motif syllables, had zero self-repetition probability and zero bout beginning probability, while INs and calls had finite self-repetition and bout beginning probabilities (Figure 2.1). Based on this, I wrote a custom Matlab script to classify syllables for the birds used in this study. Specifically, the script classified all syllables with self-repetition probability > 0 and bout beginning probability > 0 as INs. Other syllables were classified as motif syllables. I did not classify syllables as calls. This script was used to classify syllables from song bouts for all birds used in this study and I then calculated IN number as described below.

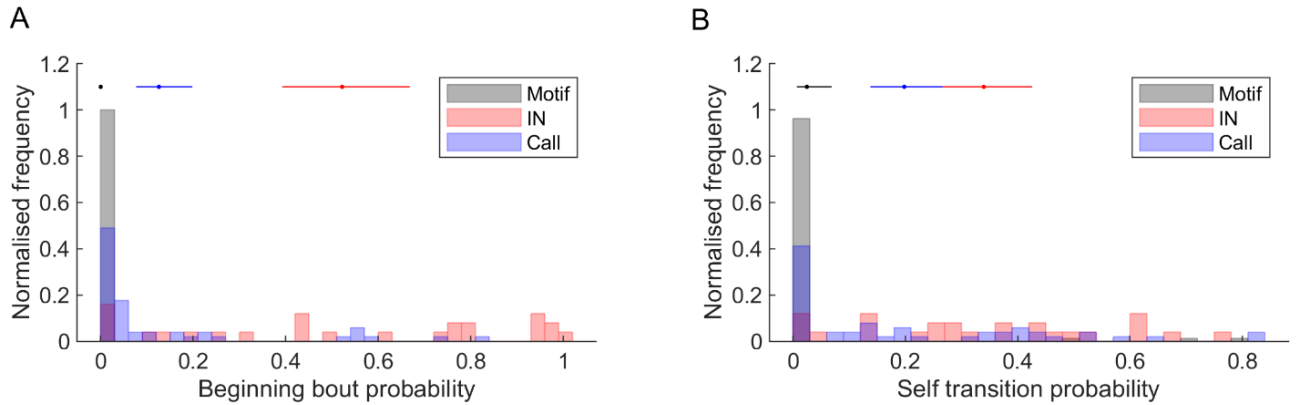


Figure 2.1 Bout beginning probability and self-transition probability distributions for identified motif syllables, introductory notes (INs) and calls for 20 unrelated birds

(A) Distribution of beginning bout probability representing the probability of a syllable occurring at the beginning of the bout. (B) Distribution of self transition probability for each syllable. In both (A) and (B), analysis was done on 157 syllables from 20 birds unrelated to the birds used in this study. Circles and whiskers at the top of both plots represent mean and 95% confidence intervals for the different categories. Colours represent different syllable types: motif syllables (black, $n=81$), INs (red, $n=25$) and calls (blue, $n=51$).

2.2.3.4 IN number calculation

Similar to earlier studies [23, 24, 43], I measured IN number at the beginning of each bout as the last set of consecutive INs, with ≤ 500 ms between them, that were produced before the first motif of the bout. To check whether the 500ms criterion influenced the results, I also counted IN number with different inter-IN intervals starting from 50ms to 750ms (in 50ms steps) as criterion, repeated the calculation of IN number in each bout and then re-calculated the correlation between mean IN number for normally reared pupils and their fathers. Correlation between mean IN number for normally reared pupils and their fathers remained significant at all criteria. The magnitude of the correlation was > 0.575 for intervals of 200ms and above. For values below 200ms, the strength of the correlation remained significant but reduced to 0.555 at 150ms, 0.509 at 100 ms and 0.429 at 50ms.

2.2.3.5 Comparison of IN acoustic structure

Acoustic structure similarity for both motifs and INs was computed using Sound Analysis Pro (<http://soundanalysispro.com/>) [44]. Twenty randomly picked motifs and 10 randomly picked INs were used for calculating similarity. Both asymmetric and symmetric measurements were used for calculating song similarity. Asymmetric does not consider the order of syllables for the final similarity value, while symmetric takes syllable order into account. Asymmetric time course similarity was calculated for motif to allow for potential differences in syllable order. For eg. if the tutor's song motif consisted of 3 equal duration syllables, a, b and c in the order 'abc', a pupil who copied all 3 syllables in the order 'abc' and a pupil who copied all 3 syllables in the order 'cab' would both be 100% similar to the tutor (Figure 2.2). Pupils who copied a subset of the 3 syllables would have a similarity proportional to the fraction of syllables copied (Figure 2.2) Symmetric, time course similarity was used for comparing IN acoustic structure as these are individual syllables. To control for this difference in similarity calculation for INs and motifs, I also calculated symmetric, time course similarity for motifs. Irrespective of the mode of similarity calculation for motifs, I found no significant correlation between IN similarity and motif similarity (or similarity of first motif syllable) across birds and their tutors (Figure 2.10, 2.11). Acoustic stereotypy of motifs in individual birds was calculated by randomly selecting 5 example motifs and calculating the mean similarity between all pairwise, symmetric similarity measurements. Here I used symmetric similarity as I wanted to take sequence and timing into consideration for self-similarity measurements. IN acoustic stereotypy was calculated in a similar manner.

For calculating chance level similarity, mean similarity value between the father of a nest and one randomly selected juvenile from each of the other nests was calculated. For birds with multiple INs, similarity was calculated for each IN type and the maximum was used to represent IN acoustic structure similarity.

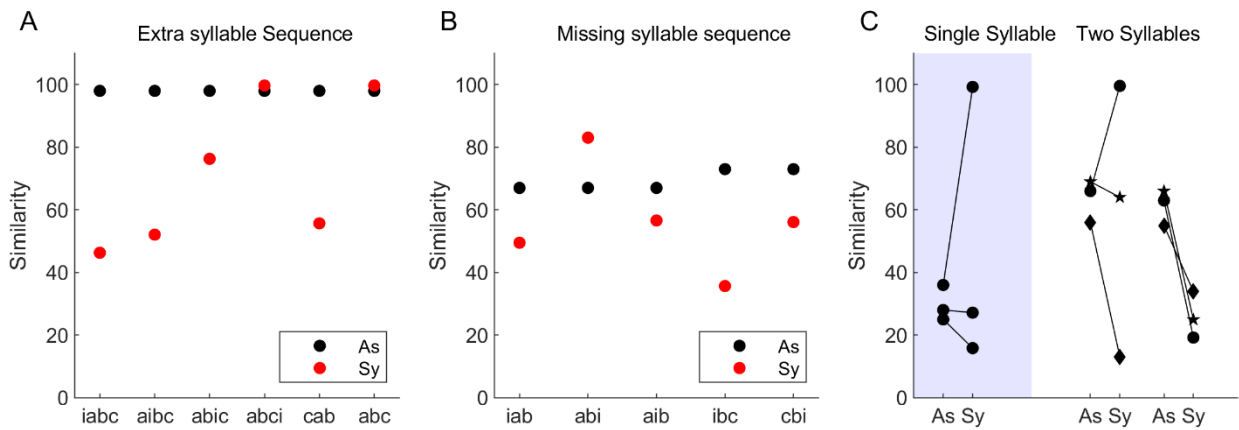


Figure 2.2 An example illustrating symmetric and asymmetric similarity measurements

All comparisons were done with a target song consisting of three syllables, a, b and c in the order abc. Asymmetric similarity measurements measure of the proportion of syllables acoustically similar to the target song independent of sequence. Symmetric similarity measurements are influenced by sequence and the identify of the first syllable in the candidate song being compared to the target song. (A) Asymmetric (black circles) and symmetric similarity (black circles) measurements for different candidate sequences of the three syllables of the target song including some with an additional introductory note (i). (B) Asymmetric (black circles) and symmetric (red circles) similarity measurements for candidate sequences that miss one of the 3 syllables of the target song. (C) Asymmetric (As) and symmetric (Sy) similarity measurements for individual syllables from the target song (Single syllable on the left) and pairs of syllables from the target song in the correct order (middle) or in the reverse order (right).

2.2.4 Statistics

Pearson correlation coefficient was used for all correlations and was calculated using the matlab function `corrcoef`. Linear fits to data were calculated using the matlab functions `polyfit` and `polyval`. Wilcoxon rank sum test was used for calculating the significance of song and IN similarity for normally reared and playback tutored birds and for comparing IN similarity of socially tutored pupils with their fathers and social tutors. Wilcoxon sign rank test was used for comparing song similarity of socially tutored pupils with their fathers and their social tutors. In case of correlation between IN and song properties, In the case of correlation between IN and song properties, Bonferroni correction was applied to control for type 1 error, while performing multiple comparisons. In figure 2.7, 2.8 alpha value was considered 0.008 (0.05/6, corrected for 6 comparisons), figure 2.10 it was 0.025 (0.05/2, corrected for 2 comparisons), and figure 2.11 it was considered as 0.012 (0.05/4, corrected for 4 comparisons).

2.3 Results

2.3.1 IN acoustic structure and mean IN number are learned from a tutor

To examine the degree to which IN acoustic structure and mean IN number are learned, I first compared these properties for birds that had been reared normally with their fathers ($n=16$ nests, $n=65$ birds, Figure 2.3C, Figure A1). INs (and song) of fathers were acoustically more similar to those of their sons as compared to unrelated birds (Figure 2.3D; $p = 0.0094$ for INs and $p < 0.001$ for song, Wilcoxon rank sum test). Mean IN number before song was also

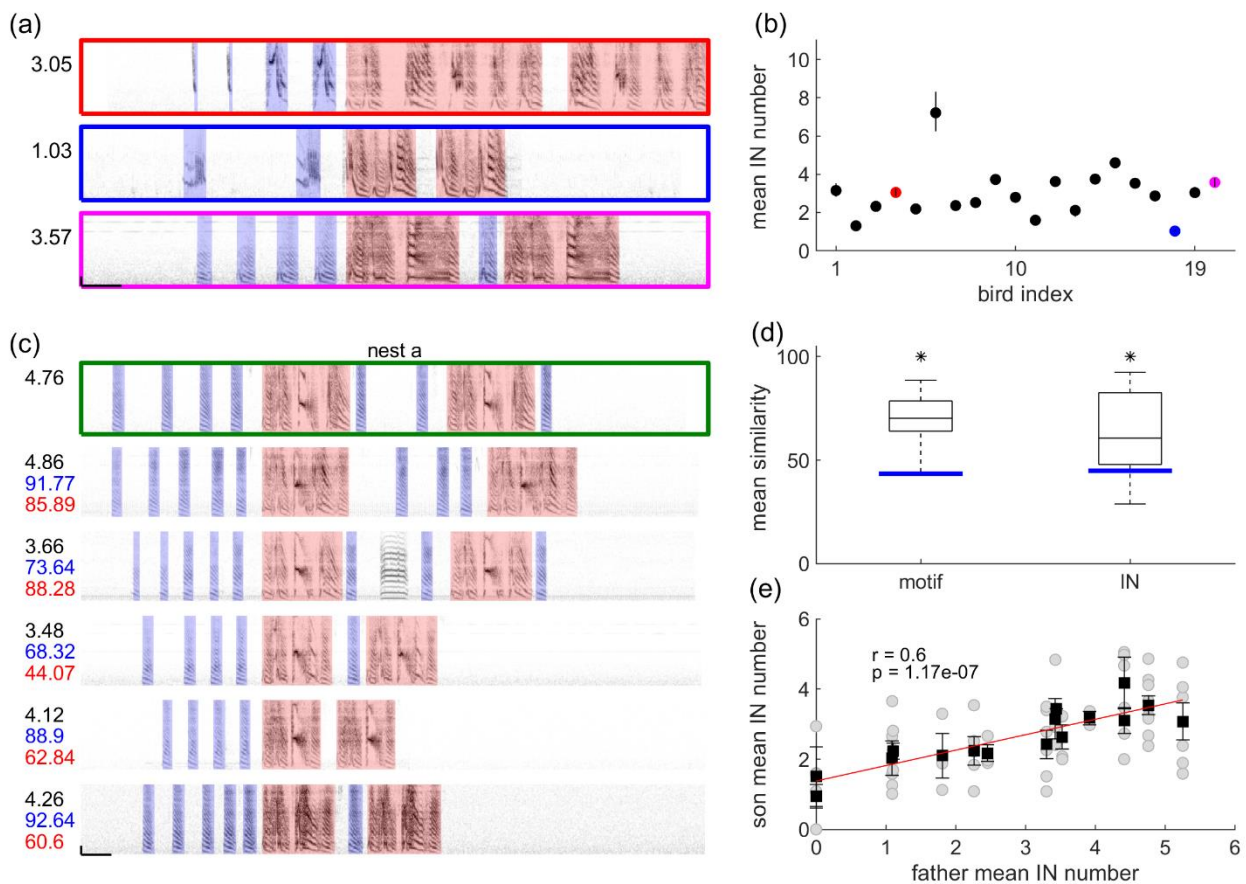


Figure 2.3 Mean IN number and IN acoustic structure are correlated between fathers and their normally reared sons

(A) Spectrograms of song bouts of 3 different zebra finches. Red shading highlights song motif and blue shading highlights INs. (B) Mean IN number varies considerably across 20 unrelated birds. Circles and whiskers represent mean and 95% confidence intervals for individual birds. Colours represent birds from (A). (C) Example spectrograms of one song bout of a father (green box) and 5 sons showing introductory notes (blue shaded boxes) and common motifs for each bird (red shaded boxes). Black numbers on the left indicate mean IN number for that bird. For sons, blue and red numbers represent mean similarity to father's INs and motif respectively. (D) Mean song similarity and maximum IN similarity between fathers and sons. Blue line represent chance level similarity (mean similarity between father from one nest and pupils from other nests). * represents $p < 0.05$, Wilcoxon rank sum test (E) Mean IN number of father vs. mean IN number of son. Grey circles represent individual birds, black squares and whiskers represent mean and s.e.m. for individual nests. Red line represents regression line.

significantly correlated between fathers and their sons (Figure 2.3E). This correlation in mean IN number was not influenced by removal of individual nests from the analysis (Figure 2.4A) and was significantly different from the correlations obtained by randomly re-assigning individual birds to different nests (Figure 2.4B).

Further, this correlation in mean IN number was significant even when syllables in individual birds were categorized into INs or song syllables based on pre-set rules (Figure 2.4C, see Methods). These results showed that mean IN number before song and IN acoustic structure were correlated between fathers and their sons.

To further test the contribution of learning to IN repetition and structure, I used a social-rearing paradigm where juvenile zebra finches were kept in visual and acoustic contact with an adult male different from their father [38,39,41] (Fig. 2.5A; $n=14$ birds; see Methods). Importantly, both IN acoustic structure and mean IN number for the social tutor were different from those of the juvenile's biological father (see example in Fig. 2.5B and Methods). INs of such socially-

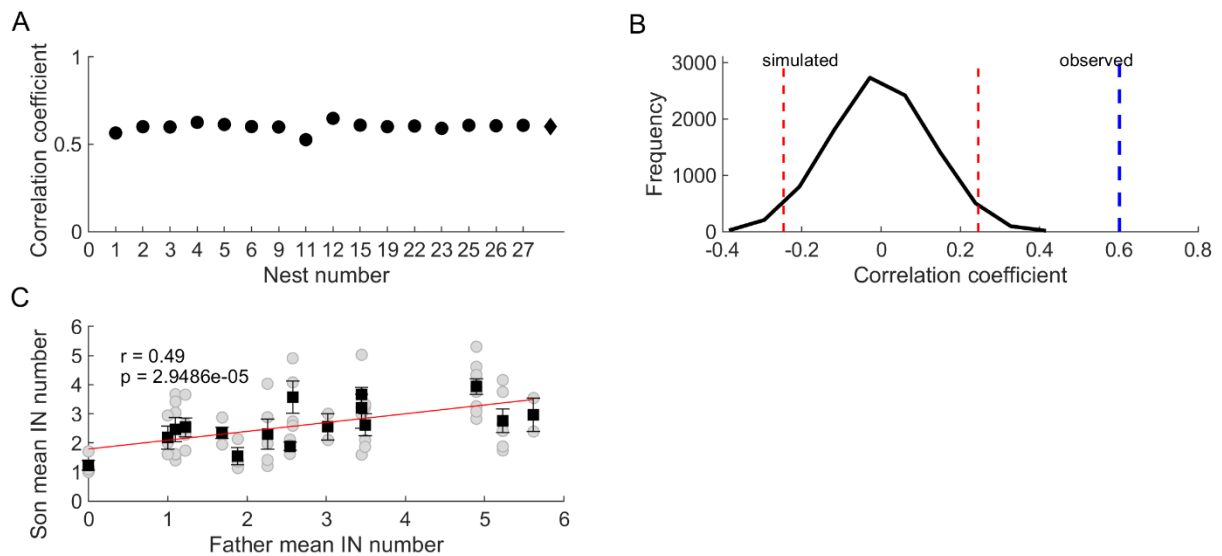


Figure 2.4 Control analyses validating observed correlation in mean IN number between fathers and their sons

(A) Circles represent correlation co-efficients obtained by removing one nest at a time from the original data. Diamond represents observed correlation coefficient calculated with all nests. All co-efficients were significant ($p < 0.05$, Pearson's correlation co-efficient). (B) Distribution of correlation coefficient values obtained by randomly re-assigning sons to different nests 10,000 times while maintaining the same number of birds for each nest from the actual data. Blue dashed line represents the correlation coefficient calculated using the actual data. Red dashed lines represent the 95% confidence interval. (C) Mean IN number of father vs. mean IN number of son calculated after using pre-set rules to categorise syllables into INs and motif syllables (see Methods). Grey circles represent individual birds, black squares and whiskers represent mean and s.e.m. for individual nests and red line represents regression line.

tutored birds were acoustically more similar to those of their social tutors than those of their fathers (Fig. 2.5C; $p = 0.028$ for INs, Wilcoxon rank sum test; $p < 0.001$ for motif, Wilcoxon sign-rank test). Mean IN number before song was also correlated with mean IN number of the social tutor but not of the father (Fig. 2.5D, 2.5E).

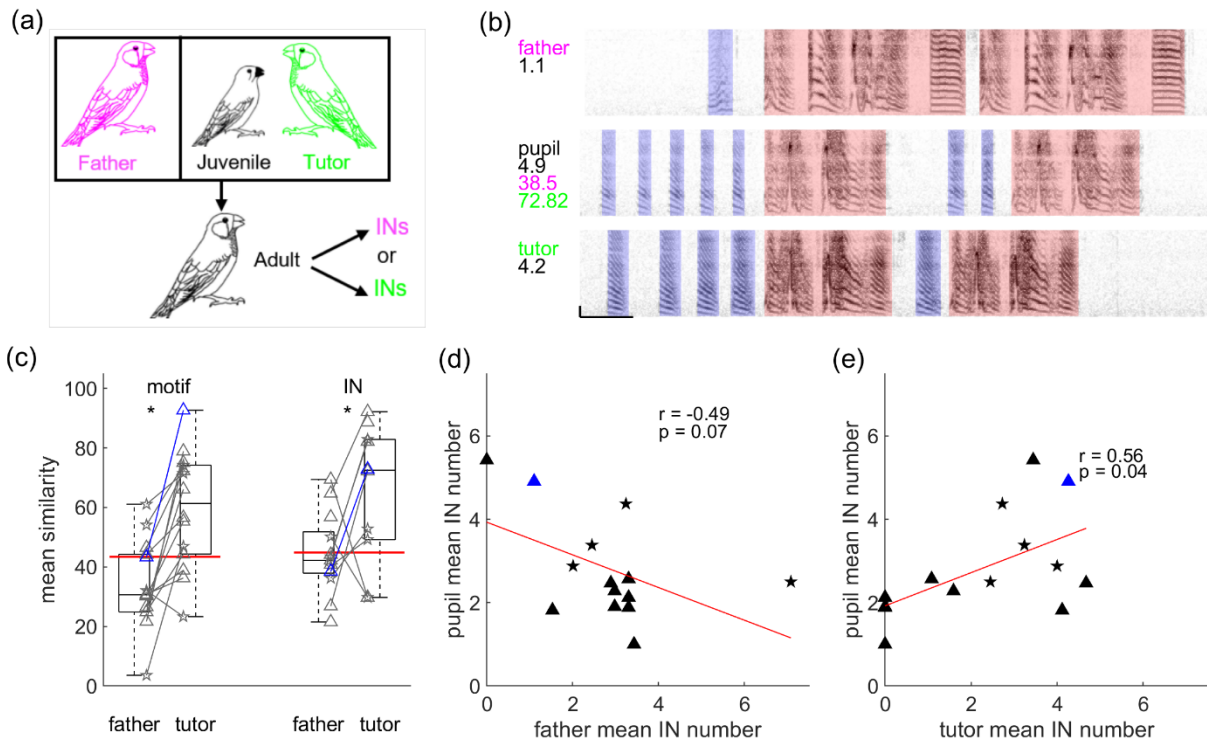


Figure 2.5 Mean IN number and IN acoustic structure, for socially reared birds, is correlated with social tutor, not father

(A) Schematic of social rearing paradigm. (B) Example spectrograms of one song bout of father (top), pupil (middle) and social tutor (bottom) showing introductory notes (blue shaded boxes) and common motifs for each bird (red shaded boxes). Black numbers on the top left side of each spectrogram represent mean IN number for that bird. For the pupil, magenta and green numbers on the left represent mean similarity to father's and social tutor's INs respectively. (C) Mean song motif similarity and maximum IN similarity of pupils to their social tutors and their fathers. Red line represents chance level similarity. * represents $p < 0.05$, Wilcoxon sign-rank test (song motif similarity) or Wilcoxon rank sum test (IN similarity) (D), (E) Mean IN number of pupil vs. mean IN number of father (D) and social tutor (E). In (C), (D) and (E), symbols represent individual birds, stars represent birds tutored at McGill, triangles represent birds tutored at IISER Pune. Red line represents regression line. Blue triangle in (C), (D) and (E) represents bird shown in (B).

The correlation with social tutor was significantly different from that obtained by randomly re-assigning birds to different social tutors (Figure 2.6A, 2.6B) and was significant even when syllables were categorized based on pre-set rules (Figure 2.6C, 2.6D). These two sets of results

indicate that the number and acoustic structure of INs are learned from a tutor (father for normally reared birds and social tutor for socially-reared birds).

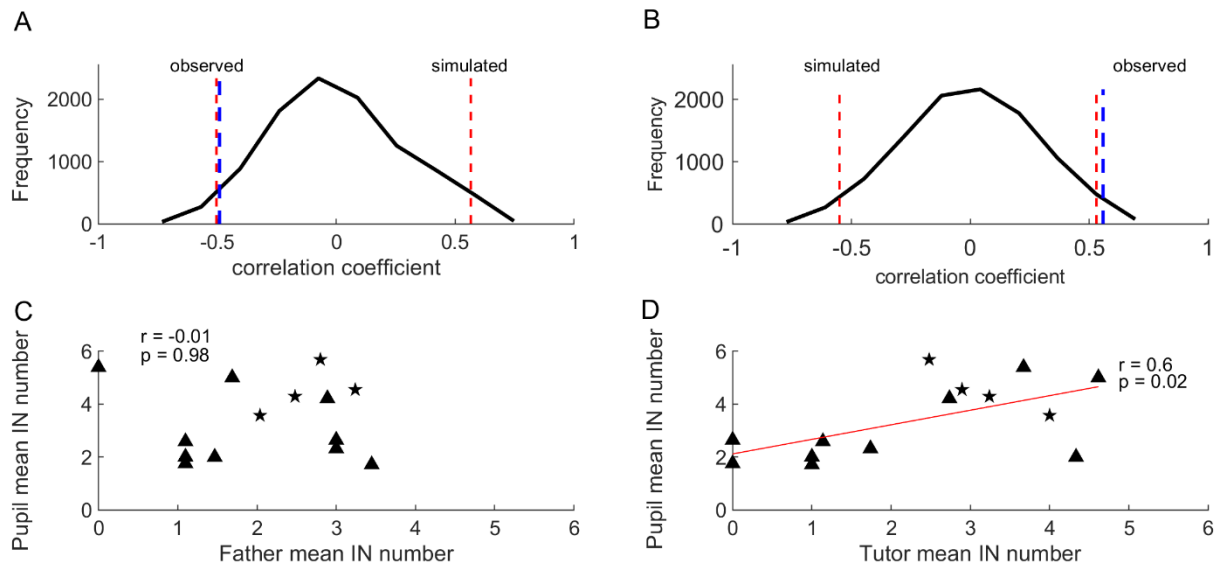


Figure 2.6 Control analyses validating observed correlation in mean IN number between pupils and their social tutors

(A) Distribution of correlation coefficient values obtained by randomly re-assigning pupils to different father / social tutor combinations 10,000 times. Correlation between pupil and father (left) and correlation between pupil and social tutor (right). Blue dashed line represents the correlation coefficient calculated using the actual data. Red dashed lines represent the 95% confidence interval. (B) Mean IN number of father vs. mean IN number of pupil (left) and mean IN number of social tutor vs. mean IN number of pupil (right) calculated after using pre-set rules to categorise syllables

2.3.2 Accurate copying of INs is independent of accurate copying of song

Previous studies have suggested that INs represent motor preparation before song [23,24]. The motor preparation hypothesis predicts correlations between properties of INs and properties of songs that follow. For example, the mean number of IN repetitions could be correlated with specific features of song. Across all birds, I compared a number of song features with the mean variability in number of INs (Figure 2.7, Figure 2.8) and didn't find significant correlations following Bonferroni adjustment ($\alpha = 0.008$; $0.05/6$).

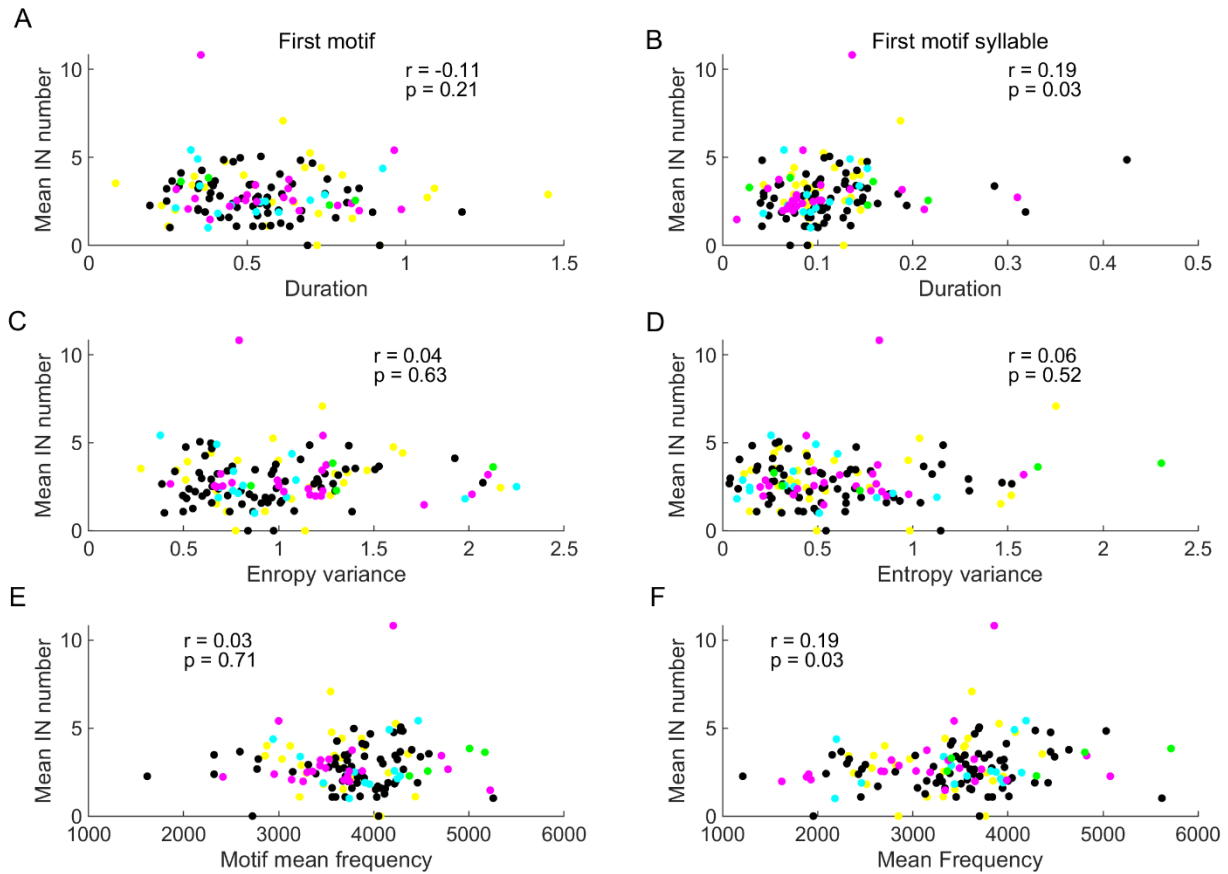


Figure 2.7 Variation in IN number is not correlated with variation in song

(A), (B), (C), (D), (E) and (F) Mean IN number across birds vs. first motif duration (A), first motif syllable duration (B), mean entropy variance of entire motif (C), mean entropy variance of first motif syllable (D), mean frequency of entire motif (E) and mean frequency of first motif syllable (F). Circles in all plots represent individual birds. Colours represent different groups; black represents normally reared, cyan represents socially reared, magenta represents playback tutored, green represents isolates and yellow represents fathers/tutors. For (A), (C) and (E), only birds with stereotyped first motifs were included ($n=129$). For (B), (D) and (F) all birds were included ($n=132$).

I also compared a number of song acoustic features with the acoustic features of INs and stereotypy of IN acoustic structure (Figure 2.9 and Figure 2.8). Among all these features, I only found weak correlations for mean frequency of INs and mean frequency of songs (Figure 2.9A). These results suggested that IN features were only weakly correlated, if at all, with features of song.

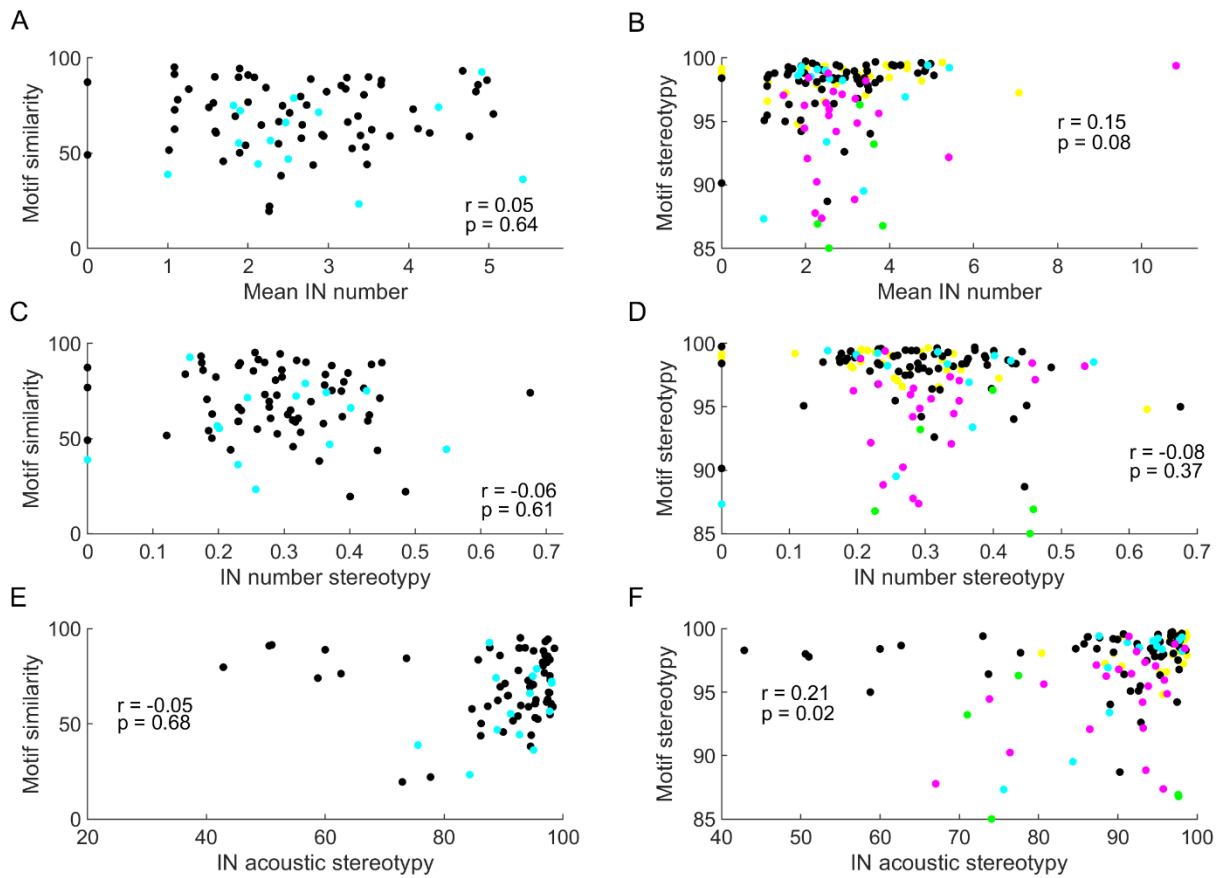


Figure 2.8 Mean IN number, degree of stereotypy of IN number and IN acoustic stereotypy show weak or no correlations with motif similarity to tutor and motif stereotypy

(A), (B) Mean IN number vs. motif similarity to tutor (A) and motif stereotypy (B). (C), (D) IN number stereotypy (CV of IN number) vs motif similarity (C) and motif stereotypy (D). (E), (F) IN acoustic stereotypy vs motif similarity (E) and motif stereotypy (F). Circles in all plots represent individual birds. Colours represent different groups; black represents normally reared, cyan represents socially reared, magenta represents playback tutored, green represents isolates and yellow represents fathers/tutors. (A), (C) and (E) includes only normally and socially reared birds ($n=79$). (B) and (D) include all birds ($n=131$; the father of one playback tutored bird was not included because of variable motif and very few song files). (F) includes only birds with INs ($n=127$). For birds with multiple types of INs, IN acoustic stereotypy is the average of acoustic stereotypy of each IN type.

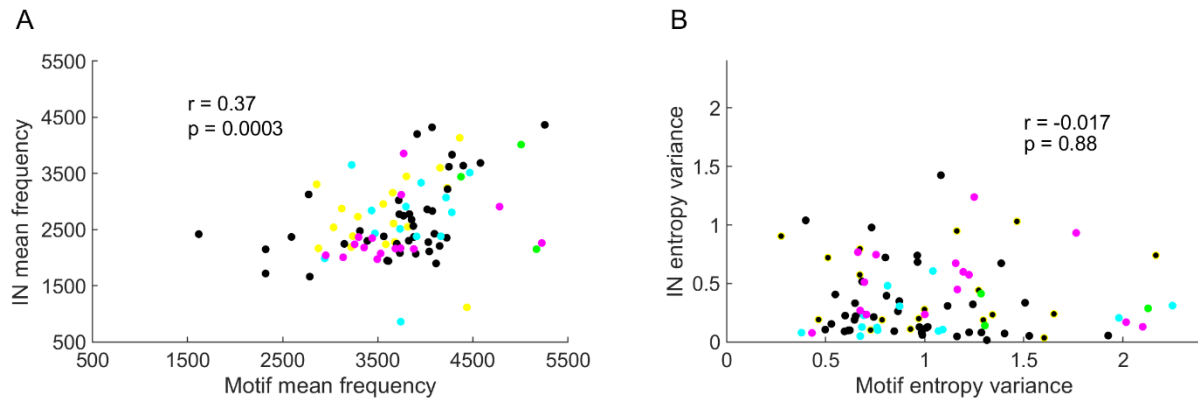


Figure 2.9 Correlations between IN acoustic properties and motif acoustic properties

(A) IN mean frequency vs Motif mean frequency. (B) Complexity of INs (measured by entropy variance of INs) vs complexity of the motif (mean entropy variance of the motif). Circles in all plots represent individual birds with one type of IN ($n=90$). Colours represent different groups; black represents normally reared, cyan represents socially reared, magenta represents playback tutored, green represents isolates and yellow represents fathers/tutors.

The motor preparation hypothesis also predicts similar INs before similar songs representing equivalent amounts of preparation. Therefore, if a pupil copied his tutor's songs accurately, he would also end up producing INs similar to the tutor. To rule out this possibility, I compared the accuracy of song imitation with the accuracy of IN imitation. Accuracy of song imitation was measured with Sound Analysis Pro [44] using asymmetric mode. Asymmetric mode measures acoustic similarity between the tutor song and the song of the pupil without taking syllable sequence into consideration (see Methods and Figure 2.2). I chose asymmetric mode to compare acoustic similarities between the song of the tutor and pupil without regard for the sequence. Symmetric mode comparisons were used for comparing INs as these are single syllables and there is no sequence comparison involved. Across all normally reared and socially reared birds, the degree of song similarity or the degree of similarity of the first song syllable between birds and their tutors was not correlated with similarity in IN number (Figure 2.10A, 2.11A, 2.11C) or similarity in IN acoustic structure (Figure 2.10B, 2.11B, 2.11D) between birds and their tutors (father or social tutor). Overall, these results confirmed that the correlations seen in mean IN number and IN acoustic structure between tutor and tutor-reared pupil were a result of learning and not a consequence of INs functioning as motor preparation for song.

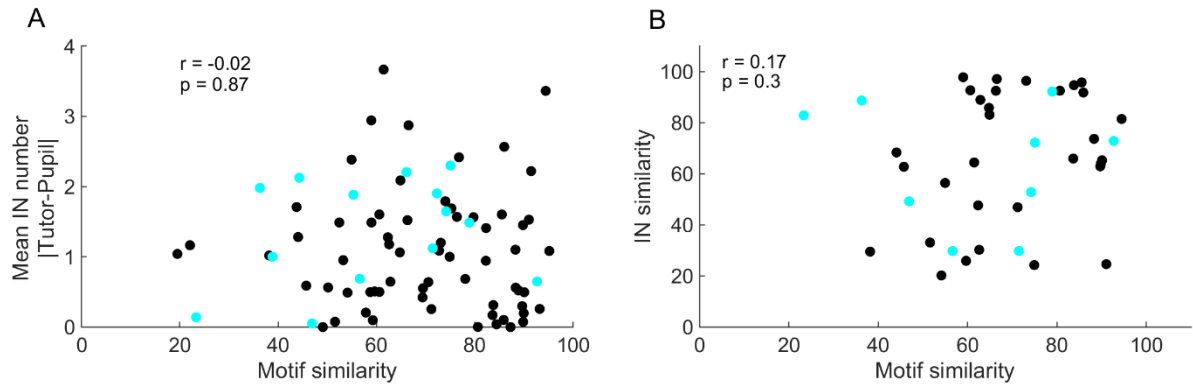


Figure 2.10 Accuracy of IN learning is not correlated with accuracy of song learning

(A), (B) Motif similarity between tutor (father or social tutor) and pupil vs. absolute difference in mean IN number between tutor and pupil (A) or IN similarity between tutor and pupil (B). Here motif similarity and IN similarity were calculated using asymmetric, time-course similarity and symmetric, time-course similarity respectively. Cyan circles represent socially reared birds and black circles represent normally reared birds. Only cases where both tutors

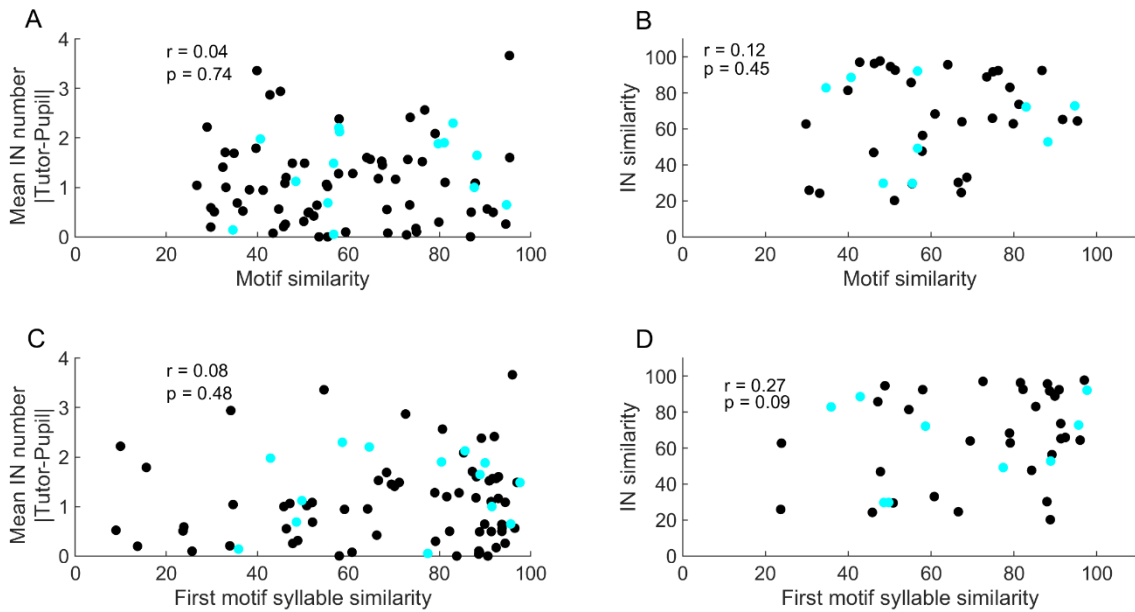


Figure 2.11 Accuracy of IN learning is not correlated with accuracy of song learning

(A), (B) Motif similarity between tutor (father or social tutor) and pupil vs. absolute difference in mean IN number between tutor and pupil ($n=79$) (A) or IN similarity between tutor and pupil ($n=40$) (B). Here motif similarity and IN similarity were both calculated using symmetric, time-course similarity. (C), (D) First motif syllable similarity between tutor (father or social tutor) and pupil vs. absolute difference in mean IN number between tutor and pupil ($n=79$) (C) or IN similarity between tutor and pupil ($n=40$) (D). In all panels, cyan circles represent socially reared birds and black circles represent normally reared birds. For (B) and (D), only cases where tutors and pupils had only one type of IN were included.

2.3.3 Biological predispositions in IN production and learning

My results suggest that INs are learned from a tutor just like birds learn their song motifs from their tutor. Previous studies have demonstrated the presence of biological predispositions in zebra finch song learning [34,35,45]. For example, juvenile zebra finches that are individually tutored with random sequences of syllables converge on similar motif sequences [34,35]. To identify biological predispositions, if any, in IN production, I experimentally tutored juvenile zebra finches with songs that lacked INs (Figure 2.12A, n=22, see Methods for details of tutoring). Despite being tutored without INs, juveniles tutored in this manner produced 3 INs, on average, before their songs (Figure 2.12B for an example, Fig. 4E). The number of these

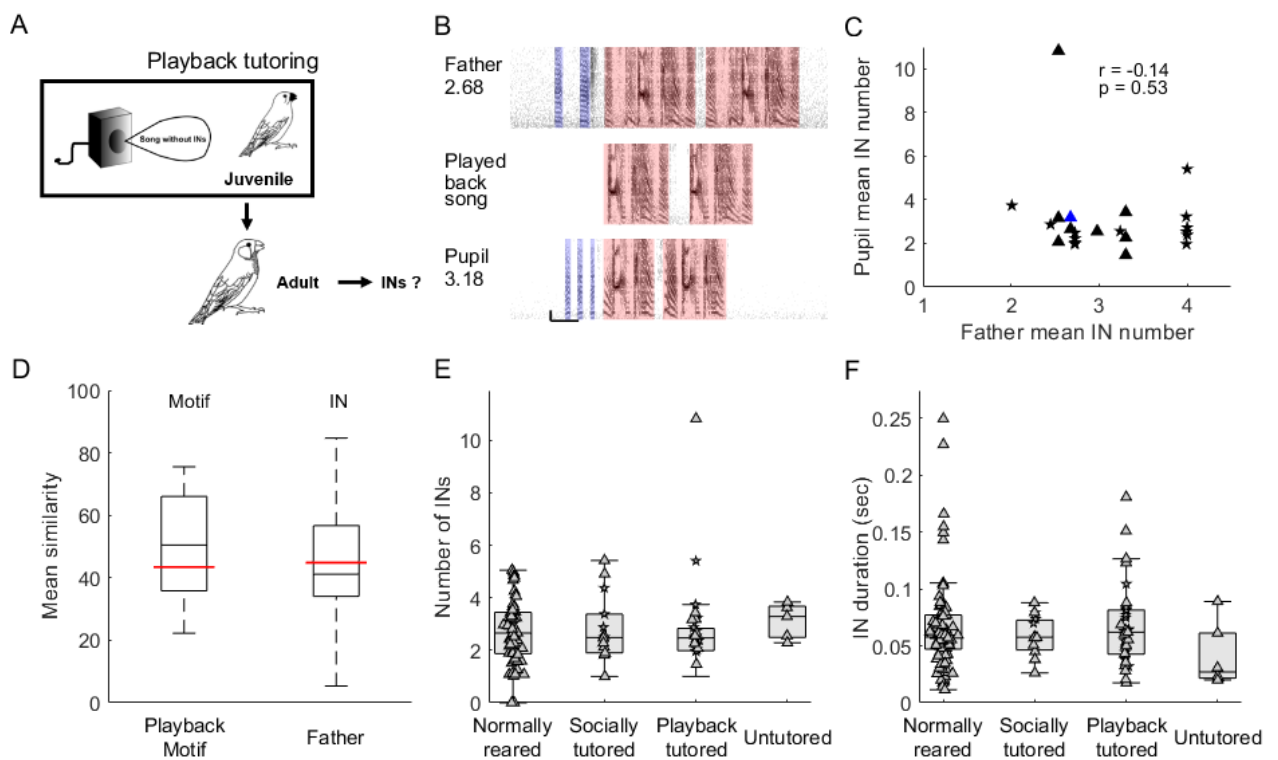


Figure 2.12 Biological predispositions in IN production

(A) Schematic of playback tutoring paradigm. (B) Example spectrograms for a song bout for the father (top), played back song (middle) and playback-tutored bird (bottom). Blue shaded regions highlight INs and red shaded regions highlight song motifs. Black number on the left of the spectrograms represent mean IN number for that bird. (C) Mean IN number for father vs. mean IN number for playback tutored birds. Blue triangle represents bird shown in (B). (D) Mean motif similarity for playback tutored birds relative to played back motif and mean IN similarity relative to father. Red lines represent random similarity. (E), (F) Box plot representing mean number (E) and duration (F) of INs produced by normally reared (n=65), socially tutored (n=14), playback-tutored (n=22) and untutored birds (n=5). Circles represent INs in individual birds. Multiple IN types in a given bird are represented separately, so the total number of points in each category can exceed the number of birds. In (C), (E) and (F), stars represent McGill birds and triangles represent IISER Pune birds.

INs was not correlated with those of the father (Figure 2.12C).

The acoustic similarity of these INs to those of the father was comparable to the similarity with unrelated birds (same as Figure 2.3D), showing that these INs were acoustically different from those of the father. (Figure 2.12D, $p = 0.86$ for INs and $p = 0.18$ for motif, Wilcoxon rank sum test). These results suggest more general, species-specific, predispositions in IN production rather than direct heritability from the father. Another way to reveal biological predispositions in IN production is to analyze INs in the songs of untutored birds. As observed in a previous study [10], I found that untutored birds produce INs before their songs ($n=5$, see Methods for details of untutored birds). Interestingly, the mean number of such INs produced before song (Fig. 2.12E; $p = 0.57$, one-way ANOVA) and the duration of these INs (Figure 2.12F; $p = 0.36$, one-way ANOVA) was similar across all categories of birds, irrespective of tutoring experience. Other features of INs differed between birds experimentally tutored without INs and birds normally or socially reared (Figure 2.13) highlighting the role of learning. Thus, on average, normally reared, socially reared, operantly tutored and untutored birds produced three, 60ms long, INs before starting their songs (Figure. 2.12E, 2.12F). This bias to produce ~ 3 INs

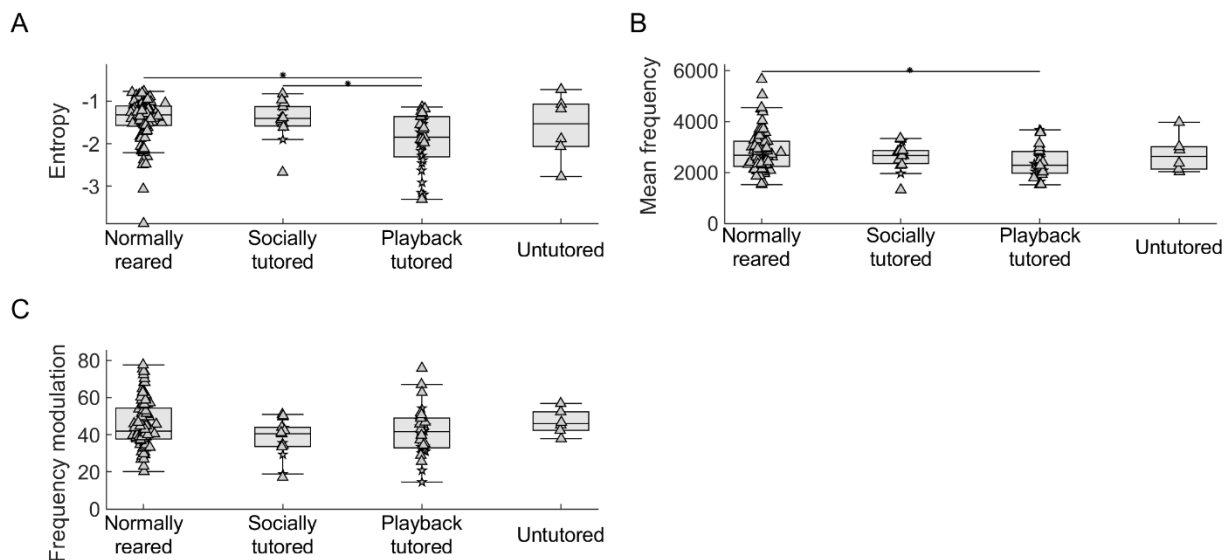


Figure 2.13 Differences in acoustic properties of INs across zebra finches with different tutoring experiences

(A), (B), (C) Box plot representing entropy (A), mean frequency (B) and frequency modulation (C) of INs produced by normally reared ($n=65$), socially tutored ($n=14$), playback-tutored ($n=22$) and untutored birds ($n=5$). Circles represent INs in individual birds. Multiple IN types in a given bird are represented separately, so the total number of points in each category can exceed the number of birds. * represents $p < 0.05$ for the post-hoc Tukey-Kramer test after one-way ANOVA. One-way ANOVA p -values are 0.00027 (for entropy), 0.034 (for mean frequency) and 0.064 (for frequency modulation). In all panels, stars represent McGill birds and triangles represent IISER Pune birds.

before song can also be observed in the data for birds tutored with songs that contained INs.

Juveniles tutored by adults that produced, on average, < 3 INs tended to produce more INs than their tutor (Figure 2.3E, 2.5E).

On the other hand, juveniles tutored by adults that produced, on average, > 3 INs tended to produce fewer INs than their tutor (Figure 2.3E, 2.5E).

2.4 Discussion

The complex vocal displays of many songbirds begin with the repetition of simple, introductory notes (INs) before the production of their learned song. Here, I show that INs, just like elements in song, are learned and shaped by biological predispositions. Specifically, using zebra finches, I show that the mean number of times an IN is repeated and the acoustic structure of INs are learned from a tutor. This learning is independent of the accuracy of song imitation and largely independent of features of the songs that follow. Finally, I reveal the existence of biological predispositions in IN production. Both untutored birds and birds tutored with songs without preceding INs, produced INs before their songs and these INs were similar in duration to the INs produced by birds tutored with songs and INs. On average, these birds produced ~ 3 INs before starting their songs and this bias for ~ 3 INs was also visible in birds tutored with INs; i.e. if the tutor sang < 3 INs, juveniles ended up singing a little more than their tutor while if the tutor sang > 3 INs, juveniles ended up singing a little less than their tutor. Taken together, these results demonstrate that the properties of INs are also shaped by learning and biological predispositions.

2.4.1 Learning of IN number and structure

How do birds learn the number and structure of INs? Earlier studies have shown the presence of multiple strategies for learning song that involve either learning the sequence first and gradually changing the acoustic structure or learning each syllable sequentially [40,46]. Similarly, juvenile birds could potentially learn IN number first while gradually modifying IN acoustic structure. Alternatively, birds could learn the structure of INs and then gradually learn to transition to the motif after the correct number of INs are repeated. Further studies of IN development in young birds could shed more light on the process of IN learning.

2.4.2 Relation to introductory notes of other songbirds

How do INs that precede zebra finch song relate to introductory gestures before other animal behaviors? As discussed earlier, many oscine songbirds produce INs before the start of their songs [6–17]. While systematic comparisons of INs across different species have not been done, it is clear that the acoustic characteristics of INs differ across species, for eg: INs of white-crowned sparrows are long, tonal sounds [7] unlike the short, broad-band sounds typically made by zebra finches. My results showing the learning of IN acoustic properties and a biological predisposition for INs of a particular duration, provide a simple explanation for variation across species.

2.4.3 Functional significance of INs and other such introductory gestures

What is the functional role of INs in zebra finch song? As discussed earlier, INs have been hypothesized to enhance the communicative value of song by “alerting” the receiver [19,20] or by providing “context” to the song [21,22]. INs have also been hypothesized to function as motor preparation [23,24]. My data showing learning of IN number and IN acoustic structure suggests that these two aspects may not reflect motor preparation. Infact, contrary to the predictions of the motor preparation hypothesis, I found only weak correlations, if at all, between IN properties and features of the songs that follow. My current results showing the learning of mean IN number and IN acoustic structure suggest that these two features could provide context to the song in the form of individual, regional or species identification. Learning would provide a reliable way to generate variation within specific populations and transmit this information across generations.

However, my data also reveals biological predispositions in IN production. Specifically, I observed that zebra finches are biased to produce short-duration vocalizations as INs, approximately three times before their songs, regardless of their tutoring experiences during development. All my experimentally tutored birds were isolated from their father very early in life and such short exposure to the father is not enough for song learning [47]. However, given that zebra finch embryos, in the egg, also learn some characteristics of vocalizations [48,49], my results do not completely exclude the role of a very early component of learning, in the production of repeated syllables (INs) at the start of a song bout. Interestingly, INs are also seen before the start of song in suboscine birds [13,15,17] where song is not learned and before advertisement calls in other vertebrates that produce unlearned vocalizations including frogs [4] and toadfish [5]. More generally, introductory gestures are also seen before non-vocal

displays like the head-bobbing displays of lizards [2,3] and my results suggest the possible presence of conserved biological predispositions in the production of introductory gestures.

A common feature of many of these introductory gestures is repetition. Such repetition is also a feature of a number of human behaviors. For example, skilled motor behaviors, especially as part of sports, are preceded by repetitive “pre-performance” routines, like ball-bouncing before a tennis serve [50]. Repetition is also a part of motor stereotypies in normal children and is often accentuated in disorders like autism [51,52]. However, these motor stereotypies are slower (lasting seconds to minutes) [51] when compared to INs or pre-performance routines that are seen in sports, suggesting less similarities with INs. What might be the function of this repetition? One possibility is motor preparation. Consistent with this idea, pre-performance routines, like ball-bouncing before a basketball free throw, appear to correlate with accurate performance; following a consistent pre-performance routine may be linked with greater accuracy with the free throw [53,54]. This suggests the possibility that INs could also have a similar function. Further experiments disrupting INs using electrical/optogenetic stimulation would help better understand whether IN repetition is necessary for song initiation.

Overall, my results demonstrate that mean number and acoustic structure of INs that precede zebra finch song are learned from a tutor, while the production of repeated INs before song is a biological predisposition. These results provide a novel perspective on understanding introductory gestures that are widely prevalent before animal movements. Both communicative [19–22] and motor preparatory functions [23,24] have been hypothesized for these gestures, albeit, separately in different animal models and my data suggests that both functions can be served by the different attributes of introductory gestures. Irrespective of their function, a combination of learning and biological predispositions, as demonstrated by my results, suggests simple mechanisms for generating variation in introductory gestures between individuals, while maintaining the species-specific structure of complex displays, like birdsong.

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Chapter 3

Characterize innate/learned properties of introductory notes

Logan S. James, Jon T. Sakata provided isolates and playback tutored birds data; Mimi Kao and Satoshi Kojima provided data for other isolates data.

3.1 Introduction

Animals communicate with their conspecific using different displays, which can be vocal or visual displays (Bradbury & Vehrencamp, 2011). Many of these communicative displays begin with introductory gestures (Backwell, 1988; Fleishman, 1992; Otter, 1996; Sossinka & Böhner, 1980; Thorson & Fine, 2002). Some examples of animals that start their acoustic display with introductory gestures include songbirds, which begin their songs with introductory vocalizations (Richards, 1981; Sossinka & Böhner, 1980); frogs (Backwell, 1988; Littlejohn & Harrison, 1985) and toadfish (Thorson & Fine, 2002) produce introductory vocalizations before their advertisement calls. Though oscines are well known for their song learning, initiation of song with introductory notes has been reported in suboscine birds, where the song is not learned (Chelén et al., 2005; Kroodsma et al., 1987; Seddon & Tobias, 2006). Other than acoustic, visual displays like head-bobbing by lizards also start with introductory tail flicks/ push-ups (Fleishman, 1992; Peters & Evans, 2003). The presence of introductory elements in different animals suggests that introductory gestures might play an essential role in communication, or they might be required to produce the final acoustic or visual display. Given the presence of introductory gestures in different displays, different functions have been suggested for these introductory gestures. Thus, understanding the properties of introductory gestures/notes (INs) is important for making better hypotheses about function. For this study, I have used a zebra finch (*Taeniopygia guttata castanotis*) as a model system, which is a well-studied Australian songbird (Zann, 1996). In zebra finches, irrespective of the sensory exposure during the song learning phase, i.e., isolates (reared in the absence of a tutor) (Price, 1979), birds tutored with playback without INs (tutored artificially with zebra finch song without INs) (Kalra et al., 2021; Chapter 2), and optogenetically tutored birds (tutored by optogenetically activating the NIF-HVC terminal for different duration) (Zhao et al., 2019) all initiate song with introductory notes (INs). Further, birds that are deafened early during song learning or as adults also initiate songs with INs (Price, 1979; Rao et al., 2019). This suggests that zebra finches have a biological predisposition to initiate songs with repetition of INs. However, whether there is a difference in the properties of INs produced by birds reared in the absence of exposure to tutor or INs, compared to normally reared birds, is not known. I have shown earlier that the juvenile learns the number and acoustic structure of INs from the tutor. Thus, like the song, which has both learned and innate properties, some properties of INs are also influenced by learning (Chapter 2; Kalra et al., 2021). Apart from number and structure, other properties of INs include temporal

and acoustic progression. INs become more similar to the last IN in the sequence toward the motif (acoustic progression). Also, INs show a temporal progression; i.e., the interval between successive INs reduces as it reaches the motif. This property of INs to converge to a final stage before the song suggests it might be involved in preparation (Rajan & Doupe, 2013). This function of INs in preparation is further supported by the fact that birds with ts nerve cut (nXIIts connects the brain to the syrinx) or deafened as adults also show temporal and acoustic progression (Rao et al., 2019). This suggests that male zebra finches do not use sensory feedback for acoustic or temporal progression; rather, it is controlled by the brain without external cues. Further, I have shown that the range of the number of INs produced by birds reared in the absence of exposure to a tutor during the learning phase is the same as for birds reared in the presence of a tutor (Chapter 2; Kalra et al., 2021). However, I did not compare properties like acoustic and temporal progression. Here, I have characterized the learned and innate properties of INs. If INs are learned vocalization similar to the song, one would expect it to be structurally different from the other innate vocalization-like calls. Thus, I have also compared INs produced by birds reared in the absence of exposure to IN with innate vocalization-like calls produced by these birds. I have characterized and compared the properties of INs in birds reared in the presence and absence of tutoring. Specifically, I have used three groups of birds, namely, (1) normally reared (reared in the presence of father), (2) playback tutored (artificially tutored by playback of zebra finch song without INs), and (3) isolates (birds reared in the absence of a tutor). Similar to a previous study, I show that birds reared without exposure to INs initiate their song with the repetition of INs (Price, 1979). This is similar to normally reared birds. Further, like normally reared birds, the interval between successive INs reduces, and INs became more similar to the last IN just before the motif. This suggests that these properties of INs are innate and might provide species specificity.

3.2 Materials and Methods

All procedures at IISER Pune were conducted after approval from the Institute Animal Ethics Committee (IAEC), IISER Pune, and were in accordance with guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), New Delhi. All procedures at McGill were conducted following approval from the McGill University Animal Care and Use Committee in accordance with the guidelines of the Canadian Council on Animal Care. All procedures at Tufts University were approved by the

Tufts University Institutional Animal Care and Use Committee. All procedures performed at UCSF were in accordance with NIH guidelines and were approved by the UCSF Institutional Animal Care and Use Committee.

3.2.1 Experimental groups

I used three different experimental groups of birds for the experiments and analyses described in this chapter. Birds that were reared in isolation or playback tutored were separated from their father at an early age (~10 days post-hatch) to prevent exposure to the father's song (see Methods Chapter 2). Following are the different groups of birds used in this study.

3.2.1.1 Normally reared birds

A total of 65 male zebra finches from 16 different nests were used. The data from these birds was used in Chapter 2 (See Chapter 2 Methods).

3.2.1.2 Playback tutored birds

22 birds were artificially tutored by the playback for the zebra finch song without INs either at McGill (n =13) or at IISER Pune (n =9). Data from these birds was also used in Chapter 2 (See Chapter 2 Methods).

3.2.1.3 Isolate birds

15 male zebra finches were reared without exposure to the zebra finch song (isolates). 7 birds were bred at IISER Pune, 3 at UCSF, 1 at Tufts University, and 4 at McGill University. Data from 5/7 IISER-bred birds is same as in Chapter 2 (see Chapter 2 Methods)

3.2.3 Data analysis

Data analysis was done using custom-written scripts in MATLAB (MathWorks). Panel function was used in scripts to generate Figures (Panel - File Exchange – MATLAB). Sigstar was used to put significance star in all figure (Rob Campbell (2023); 2013 version).

3.2.3.1 Song segmentation and annotation

Song segmentation and annotation was done in a similar way as described in chapter 2. 10 isolates that were not part of the previous chapter, silence of duration 2 seconds (n=3), 1.9 seconds (n=1), 0.5 – 1.7 seconds (n = 6) was used to define a song bout to get enough song bouts.

3.2.3.3 Self-transition

Self-transition probability is the probability of repetition for a particular syllable. The following formula was used to calculate a syllable's self-transition probability across song bouts. There was not minimum time interval between syllables considered for calculating transition probability.

$$\text{Self-transition probability for syllable "a"} = \frac{\text{Total number of a to a transitions}}{\text{Total number of a}}$$

3.2.3.4 Beginning probability

Beginning probability is the probability of a syllable to occur at the beginning (first) of a song bout. It was calculated using the following formula.

$$\text{Beginning probability for syllable "a"} = \frac{\text{Total number of occurrences of a as first syllable of the bout}}{\text{Total number of bouts}}$$

Bins size for the histogram of both self-transition and beginning probability was 0.25.

3.2.3.5 Similarity

- Structural similarity between INs and calls

Structural similarity between calls and INs produced by different isolates was calculated using Sound Analysis Pro (<http://soundanalysispro.com/>) (Tchernichovski et al., 2000). Symmetric time course measurement was used. IN/call produced by each isolate was compared to IN or call produced by other isolates. In cases where birds produced more than one type of call/IN, an average similarity value was used for the final comparison. All settings to calculate similarity were kept default except for the minimum duration which was changed to 3 msec.

- Temporal similarity

In order to calculate the temporal similarity, all vocalizations were converted to ones and silences to zeros in a song bout (Figure 3.5E). The following formula was used to calculate the correlation between two series to get the temporal similarity.

$$\text{Correlation} = \frac{(\text{sequence1} - \text{mean}(\text{sequence1})) * (\text{sequence2} - \text{mean}(\text{sequence2}))}{\text{norm}(\text{sequence1} - \text{mean}(\text{sequence1})) * \text{norm}(\text{sequence2} - \text{mean}(\text{sequence2}))}$$

norm is a MATLAB function. It calculates the Euclidean norm.

- Motif temporal similarity

In order to calculate the motif temporal similarity, the sequences of zeros and ones were aligned to the first motif syllable. A duration of 0.5 seconds from the start of the first motif syllable was considered for similarity calculation. Each bout was compared to other bouts.

- IN temporal similarity

Firstly, the sequence of zeros and ones was aligned to the last IN in the sequence. In order to make all sequences of the same length, zeros were added before the first IN (Figure 3.5E). Following this, the correlation was calculated using the formula mentioned above.

3.2.3.6 Acoustic features

I measured the following acoustic features using MATLAB code for Sound Analysis Pro (<http://soundanalysispro.com/matlab-library>). These were compared for INs produced by different groups of birds (normally reared, playback tutored, and isolates) and used for comparison with calls produced by isolates.

1. Frequency modulation: It gives an estimate of the slope of frequency in sound with respect to a horizontal line.
2. Entropy: Entropy gives a measure of noise in a sound. Its value ranges from 0 (white noise) to $-\infty$ (complete order).
3. Entropy variance: It is the variance of entropy.

4. Mean frequency: It measures the central tendency of the distribution of intensity across different frequencies present in a sound.
5. Pitch goodness: It measures the harmonic periodicity of a sound. The pitch goodness value is higher for harmonic stacks than for the modulated sound.
6. Duration: Duration is the time difference between the onset and offset of a sound.
7. Amplitude: Amplitude is a measure of the loudness of a sound.

3.2.3.7 Temporal progression

Temporal progression was calculated only for birds with mean IN ≥ 2 . INs have been previously shown to speed up towards the motif (Rajan & Doupe, 2013). In order to determine the temporal progression, the interval between successive INs was calculated (Figure 4.1B). I calculated the ratio of temporal progression for each bout and used the mean of this ratio to compare across different categories of birds. Thus, a value greater than 1 would mean the interval increases from the first IN to the last IN, while a value lower than 1 means that the interval reduces between successive INs (Figure 4.1C). Since, birds produce variable number

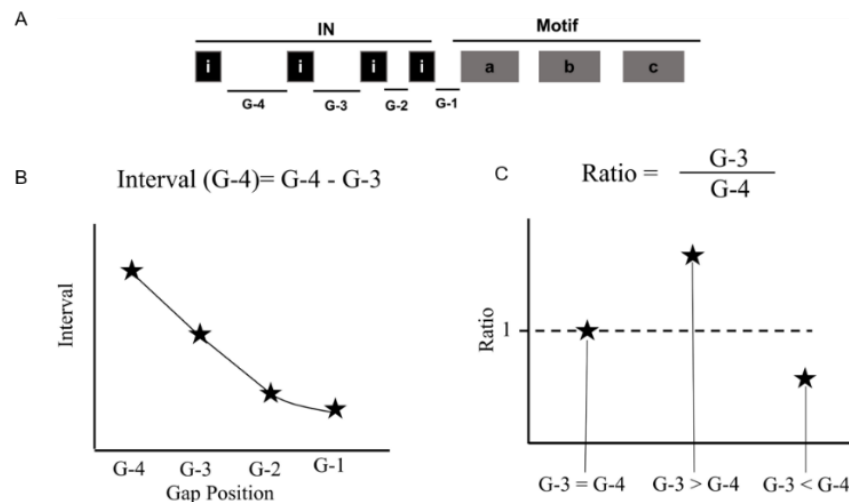


Figure3.1 Schematic for calculation of temporal progression.

A Cartoon representation of zebra finch song, 'iiii'- represents INs and 'abc' represents the motif. G-4, G-3, G-2, G-1 mark the position of interval between INs with G-4 representing interval between first and second IN, G-3 between second and third IN and so forth. B) Formula for calculating interval (top) and a cartoon plot of interval for different gap positions (bottom). C) Formula for calculating the ratio (top) and representation showing the ratio value for different relationship between the gaps (bottom).

of INs, positions where INs occurred in less 10% of bouts were excluded. Consecutive INs separated by less than 500 msec seconds interval were only considered. However, the results

didn't change after removal of 500 msec criterion and the ratio was not significantly different across groups (p-value: 0.5955, one-way ANOVA).

3.2.3.8 Acoustic progression

Acoustic progression was calculated for birds producing only one type of INs and with mean IN number ≥ 2 . In order to measure the acoustic progression, the Mahalanobis distance was calculated between each IN and the mean of acoustic features of the last IN (IN immediately before the first motif syllable). I didn't select a subset of bouts as a reference for the last as done in another study (Rao et al., 2019). Here, I have used mean value of last IN across all song bout, same way as in Rajan & Doupe (2013) to calculate the distance (Rajan & Doupe, 2013). Mahalanobis distance measures the distance between two points in 'n' dimension space. All the acoustic features mentioned above were used for its calculation. I have used mahalanobis as it is better suited for non-orthogonal space. The Mahalanobis distance is inversely proportional to the similarity of sounds, i.e., high-similarity sounds have a lower Mahalanobis distance value. The distance value was obtained using a MATLAB function called `pdist2`. Similar to temporal progression, I calculated the ratio of distance for successive INs in order to compare acoustic progression across different categories of birds. A mean of these ratio values was used to compare acoustic progression across different groups. A value greater than 1 would mean that the next IN is less similar to the last IN, while a value less than 1 would mean that the successive INs are more similar to the last IN. Similar to the temporal progression consecutive INs less 500 msec apart were considered. Here, removal of 500 msec criterion didn't affect the results and the ratio was not significantly different across groups (0.1377, one-way ANOVA). Positions where INs occurred in less 10% of bouts were excluded.

3.2.4 Statistics

All statistical tests were conducted in MATLAB. All comparisons among different groups were done using ANOVA. If the p-value was less than 0.05, Tukey-Kramer was used as a post hoc test for comparing specific pairs of groups. Matlab function `anova1` was used to perform one-way ANOVA and `multcompare` was used for post hoc tests. Paired comparisons were done using Wilcoxon signed rank, and MATLAB function `signrank` was used for this. Wilcoxon rank-sum was used to compare the similarity between calls and INs produced by isolates.

3.3 Results

3.3.1 Isolates and playback tutored birds also begin the song with repeats of introductory notes (INs) similar to normally reared birds.

Birds reared in the absence of a tutor (referred to as isolates), and birds tutored with the playback of zebra finch song without INs (referred to as playback tutored) produced song bouts that consisted of repeats of INs followed by a motif (Figure 3.2A, B, C). In all three categories of birds, INs had a high probability of occurring at the beginning of a bout and had high self-transition probability (probability of repeating syllable) (Figure 3.2D, E, F), and motif syllables rarely occurred in the beginning, and very few motif syllables had non-zero self-transition probability. The mean number of INs at the beginning of the bout and the variability in number

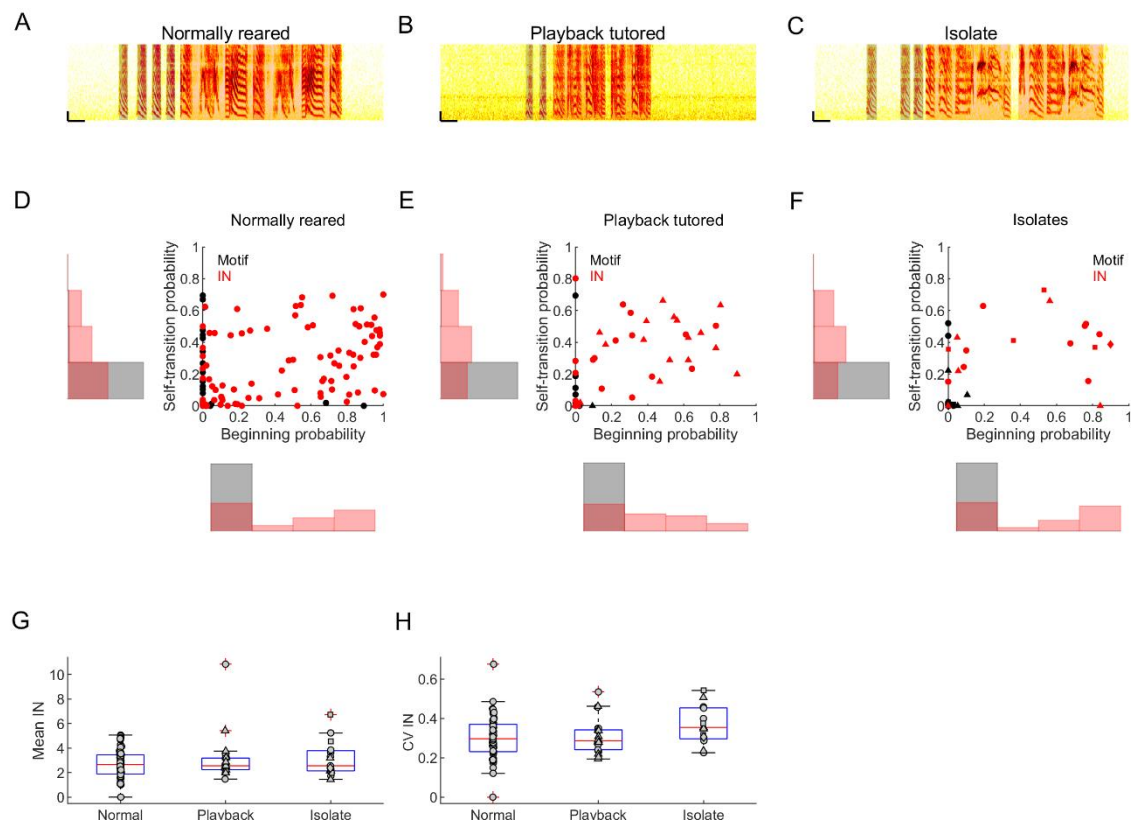


Figure 3.2 Playback tutored birds and isolates begin the song with INs.

A, B, C. Song spectrogram for normally reared, playback tutored, and isolate. The blue patch represents IN, red represents the motif. Scale bar x-axis: 150 msec, y-axis: 1000 Hz. D, E, F. Beginning vs. self-transition probability for IN (red) and motif (black) syllables for normally reared, playback tutored, and isolate, respectively. Each point represents a syllable. Marginal plots show histogram (bin size 0.25) of the same. G, H Box plot for Mean and CV of IN number for different groups (p-value > 0.05), one-way ANOVA. Different shapes represent birds from different colonies. Circle: IISER, Pune, Triangle: McGill university, Square: Korea brain research institute, Diamond: Tufts university. n = 65 (normally reared birds), n = 22 (playback tutored), n = 15 (isolates)

across bouts were comparable among three groups of birds (Figure 3.2G, H, p-value: 0.288 for mean, p-value: 0.063 for CV (coefficient of variance) of IN number, one-way ANOVA). Overall, it shows that irrespective of sensory exposure to the song during the song-learning phase, birds initiate songs with INs. This suggests the presence of an innate template in zebra finches to begin a song with INs.

3.3.2 Isolates and playback tutored birds produce INs similar in structure to normally reared birds.

Next, I examined whether the acoustic features of INs across all groups were similar. Figure 3.3 shows a spectrogram of INs for all three groups of birds. Qualitatively, INs are similar across all groups. To further characterize differences in acoustic features, I compared the acoustic properties of INs across these three groups of birds. Similar to normally reared birds, INs produced by both isolates and playback tutored birds were shorter than song syllables

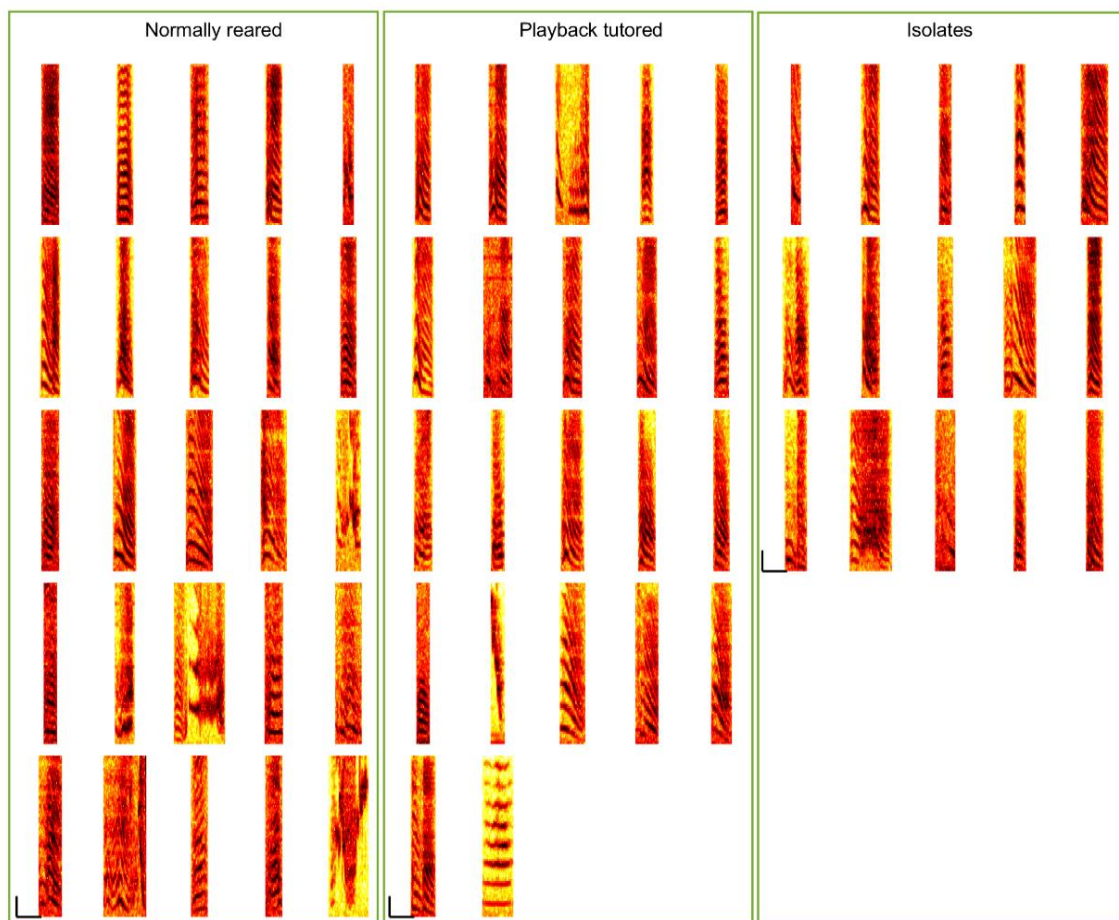


Figure 3.3 Spectrogram of INs produced by normally reared (n=25), playback tutored (n=22) and isolates (n=15). Scale bar: x-axis: 100 msec, y-axis: 1000 Hz

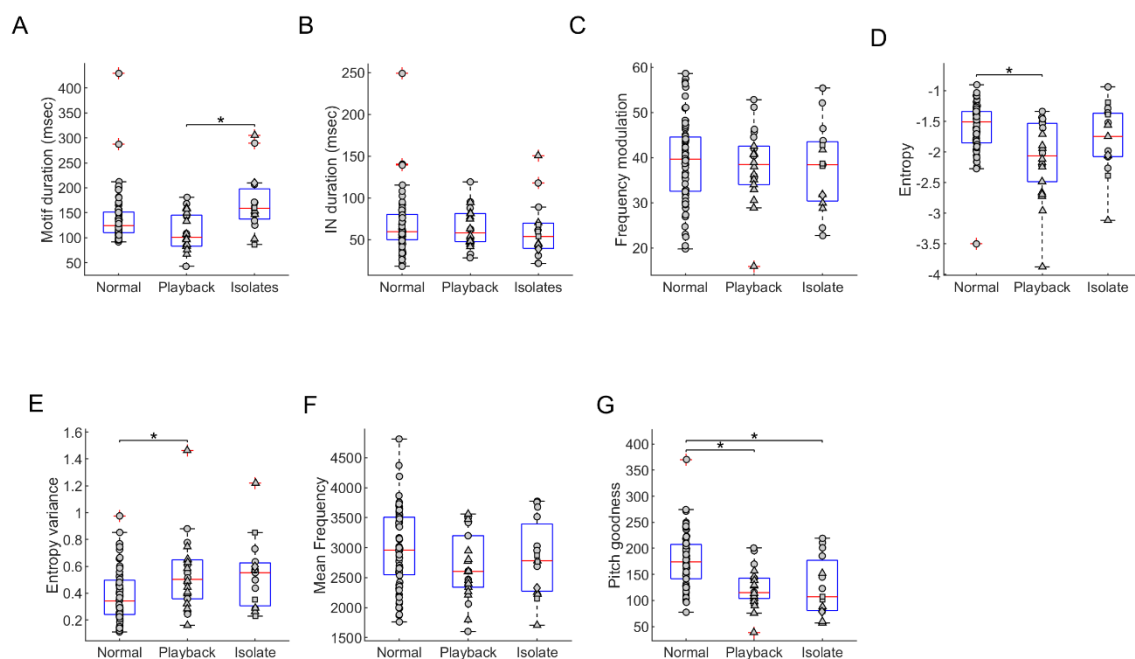


Figure 3.4 Box plot of different acoustic features of IN for different groups of birds.

Box plot for motif duration (A); Duration (B), frequency modulation (C), entropy (D), entropy variance (E), mean frequency (F), and pitch goodness (G) of INs produced by normally reared birds, playback tutored birds and isolates. Star represents $p < 0.05$, one-way ANOVA, post hoc: Tukey-Kramer. Different shapes represent birds from different colonies. Circle: IISER Pune, Triangle: McGill university, Square: Korea Brain Research Institute, Diamond: Tufts university. Each point represents a bird. $n = 63$ (normally reared), $n = 22$ (playback tutored) and $n = 15$ (isolates).

(Figure 3.4A, B, p -value < 0.001 for all groups, Wilcoxon signed rank). Also, the duration of INs produced by isolates and playback tutored was comparable to those of normally reared birds (Figure 3.4B, p -value: 0.698, one-way ANOVA). However, compared to normally reared birds, the mean motif syllable duration was shorter for playback-tutored birds and longer for isolates (Figure 3.4A), with a significant difference between isolates and playback-tutored birds (p -value: 0.026, one-way ANOVA, post hoc Tukey-Kramer). Isolates and playback tutored birds produced INs with lower pitch goodness than normally reared birds. In all other features, INs were mostly comparable across the 3 categories of birds. However, INs produced by playback tutored were different in entropy, entropy variance, and pitch goodness from normally reared birds (Figure 3.4C, D, E, F, G p -values: 0.8319, < 0.001 , 0.0068, 0.1308, < 0.001 for frequency modulation, entropy, entropy variance, mean frequency and pitch goodness, ANOVA). Overall, INs produced by playback tutored birds and isolates have lower entropy, entropy variance, mean frequency, and pitch goodness than normally reared birds. However, the difference is significant only in a few features, namely entropy, entropy variance, and pitch

goodness. The presence of a similar trend from isolates and playback tutored birds in acoustic features of INs relative to normally reared birds suggests the presence of an innate template.

3.3.3 INs acoustic properties in isolates and playback tutored birds differ from those of calls and have considerable inter-individual variability.

INs produced by normally reared birds are acoustically different from innate calls produced by these birds. This, however, could be the result of learning IN structure from the father, but what happens in the case of isolates and playback tutored birds that have not been exposed to IN during the song-learning phase? Given that both isolate and playback tutored birds have an innate tendency to initiate song with INs, I next asked if all birds previously not exposed to INs begin the song with a similar type of IN and whether they produce INs similar to calls (innate

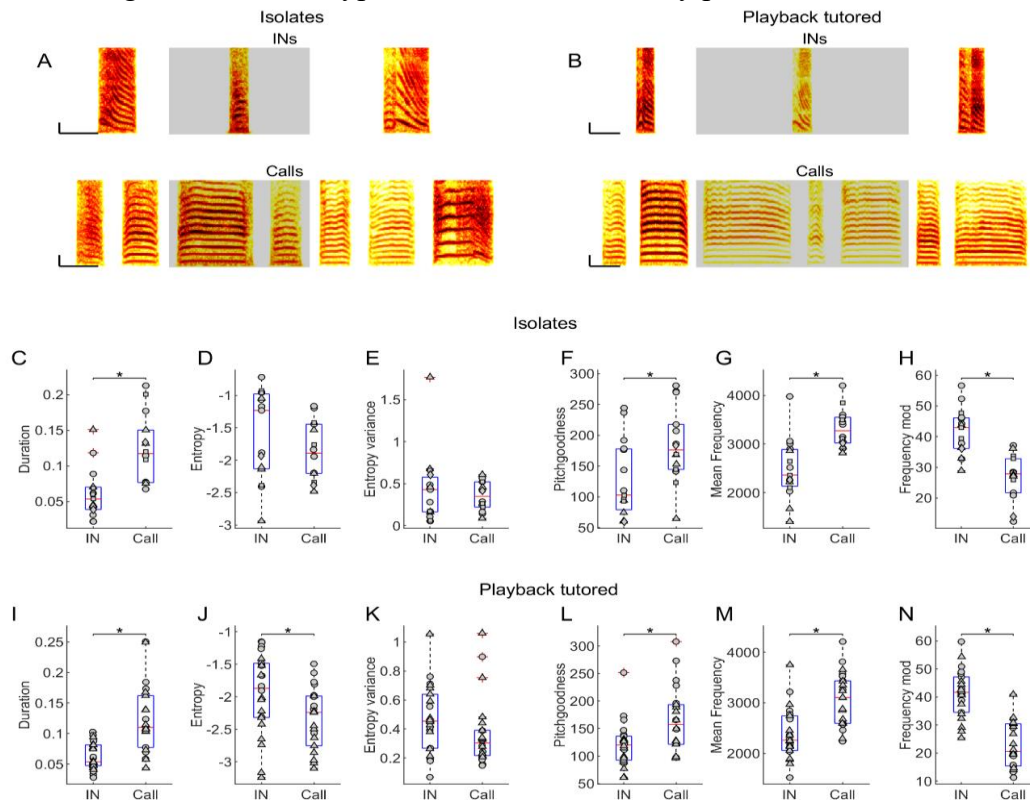


Figure 3.5 Both isolates and playback tutored birds produce INs different from calls.

A, B. Spectrogram of INs (upper) and calls (lower) for isolates and playback tutored birds. Scale bar: x-axis: 100 msec, y-axis: 1000 Hz. C, I, D, J, E, K, F, L, G, M, H, N Box plot of duration (C, I), entropy (D, J), entropy variance (E, K), pitch goodness (F, L), mean frequency (G, M), frequency modulation (H, N) for isolates and playback tutored birds. Different shapes represent birds from different colonies. Circle: IISER, Pune, Triangle: McGill university, Square: Korea brain research institute, Diamond: Tufts university. The star represents p-value < 0.05, Wilcoxon signed rank. n=22 (playback tutored), n=15 (isolates).

vocalizations used for communication). Isolates and playback tutored birds produce INs with a structure different from call (Figure 3.5A, B). Specifically, when compared to calls, INs were shorter in duration (Figure 3.5C, I, p-value 0.0023, <0.001, for isolates and playback tutored respectively, Wilcoxon signed rank), had lower pitch goodness (Figure 3.5F, L, p-value 0.0067, 0.0024 for isolates and playback tutored respectively, Wilcoxon signed rank), lower mean frequency (Figure 3.5G, M p-values 0.0023, <0.001 for isolates and playback tutored respectively, Wilcoxon signed rank) and higher frequency modulation (Figure 3.5H, N, p-value <0.001 for both isolates and playback tutored birds, Wilcoxon signed rank). Entropy was significantly higher for INs than calls for playback tutored birds (Figure 3.5J, p-value 0.0027, Wilcoxon signed rank). Isolates also show a similar trend for entropy, but this was not significantly different (Figure 3.5D, 0.2166, Wilcoxon signed rank). Furthermore, I calculated the similarity between INs and calls among birds to check for similarity across individuals for calls and INs. If INs are similar to innate vocalizations like short calls, one would expect high similarity across individuals. Alternatively, if there is no common innate template for INs, one would expect low similarity in IN structure across individuals not tutored with INs. The mean similarity of isolates for INs was 36.068 ± 1.68 , while for calls, it was 51.718 ± 3.397 (Appendix Figure A2). Thus, calls have higher inter-individual similarity than INs (p-value: 0.0016, Wilcoxon rank-sum). Overall, these results showed that INs produced by both groups of birds are structurally different among individuals, and in each individual, INs are acoustically different from innate calls. Given that the song also has an innate template and is different from calls, it suggests INs might also have an innate template different from calls.

3.3.4 Temporal progression is present in both isolates and playback tutored birds.

INs speed up and become less variable, i.e., the interval between successive INs reduces and gets less variable as it approaches the motif in normally reared birds (Figure 3.6A) (Rajan & Doupe, 2013). Next, I examined whether this was due to learning of temporal features or innate properties of INs. As seen in Figure 3.6A, (red, blue) intervals reduce and become less variable in isolates and playback tutored birds. Also, the variability of the interval reduces towards the motif. I calculated the ratio of gaps between successive INs for all groups of birds. A value less than 1 indicates that successive intervals are shorter in duration; a value greater than 1 indicates an increase in the successive intervals, while a value equal to 1 would mean no change (see methods). The ratio for all three groups of birds was comparable (Figure 3.6B p-value: 0.77, one-way ANOVA), and 31/45 normally reared birds, 14/19 playback tutored birds, and 7/12

isolates had ratios less than 1. This suggests that for most birds, irrespective of sensory exposure, the tempo of INs increases as it approaches the motif. Like normally reared birds, the last interval was shorter and more stereotyped than the first interval for isolates and playback tutored birds (Figure 3.6C, D). Given that temporal progression is maintained for INs irrespective of sensory exposure, next, I checked for temporal similarity (a measure of temporal stereotypy) across different song trials for both song and INs. In order to calculate temporal similarity, the bout was represented using zeros and ones, where zero was used to represent silence and one for vocalization. The pairwise correlation of 8 randomly selected bouts was

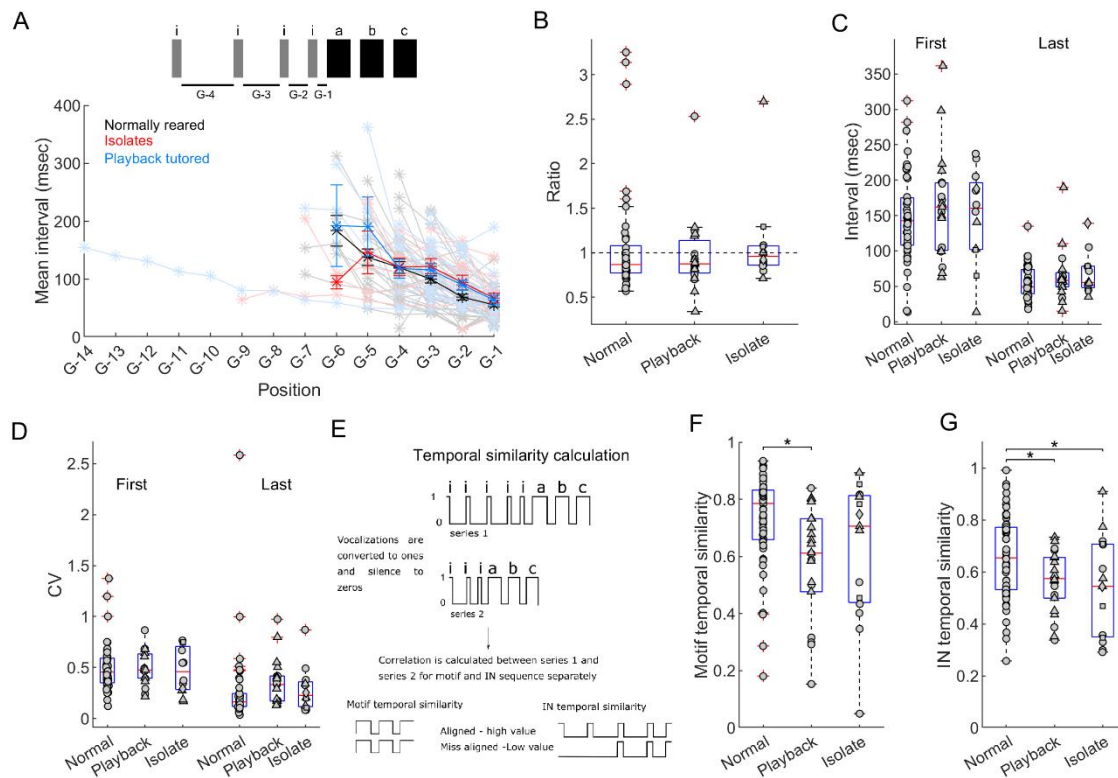


Figure 3.6 Temporal progression present in INs produced by isolates and playback tutored birds.

A Schematic representing a song (ii: INs and abc: motif) (upper panel), the plot of the interval between INs for different gap positions (Lower panel). The dark line shows the mean across birds, and the light line represents values from individual birds. Black, red, and blue colors are used for normally reared (n=45), isolate (n=12), and playback tutored birds (n=19), respectively. B. Box plot of the mean ratio of interval between successive INs. The dotted line represents a ratio equal to 1. C, D Box plot of mean (C) and cv (D) of first and last interval for all three groups. E. Schematic showing the calculation of temporal similarity. For calculating the correlation coefficient in the case of INs, the series is aligned to the last IN, and in the case of motif, it is aligned to the first motif syllable. F, G, Box plot for motif (F) and IN (G) temporal similarity for normally reared, playback tutored, and isolates. In each box plot, individual points represent different birds. Different shapes are used to represent birds from different colonies. Circle: IISER, Pune, Triangle: McGill university, Square: Korea brain research institute, Diamond: Tufts university. Star represents p-value < 0.05, ANOVA, post hoc: Tukey-Kramer.

calculated for all combinations separately of INs and motif (see methods). The sequence was aligned to the last IN for calculating the temporal similarity of INs and to the first motif syllable for calculating motif temporal similarity (Figure 3.6E). IN and motif temporal similarity for isolates and playback tutored birds were significantly lower than normally reared birds (Figure 3.6F p-value < 0.001, 3.6G p-value = 0.0092, one-way ANOVA, post hoc Tukey-Kramer). However, it was significantly different for motif only between normally reared and playback tutored birds (p-value: < 0.001, ANOVA, post hoc Tukey-Kramer). Overall, these results show that the speeding of INs before the song is independent of exposure to INs and suggests the presence of innate mechanisms for reducing the interval between successive INs. Lower temporal similarity in birds reared in the absence of exposure to song/motif suggests that song/INs produced by these birds have higher temporal variability than the normally reared birds.

3.3.5 Acoustic progression is present in isolates and playback tutored birds.

INs also have acoustic progression, i.e., successive INs become more similar to the last IN (closer to motif) in normally reared birds (Rajan & Doupe, 2013). I wanted to check if acoustic progression exists in INs produced by isolates and playback tutored birds. The acoustic progression of INs was calculated by computing the Mahalanobis distance between the last IN and INs occurring before (see methods). Similar to normally reared birds, INs produced by both groups of birds showed acoustic progression (Figure 3.7A). The mean ratio calculated from the acoustic distance of successive INs was not significantly different between groups (Figure 3.7B, p-value: 0.1982, one-way ANOVA). 21/23 normally reared birds, 10/10 playback tutored birds, and 6/8 isolates had a ratio less than 1, suggesting that INs became more similar to the last IN closer to motif onset in most birds. Also, the Mahalanobis distance was higher for the first IN than the last IN (Figure 3.7C, p-value < 0.001, 0.002, 0.015 for normally reared birds, playback tutored birds, and isolates, respectively, Wilcoxon signed rank) for all categories of birds. Similar to the temporal progression of INs, these results show that the acoustic progression is also innate and not dependent on exposure to INs during the song-learning phase.

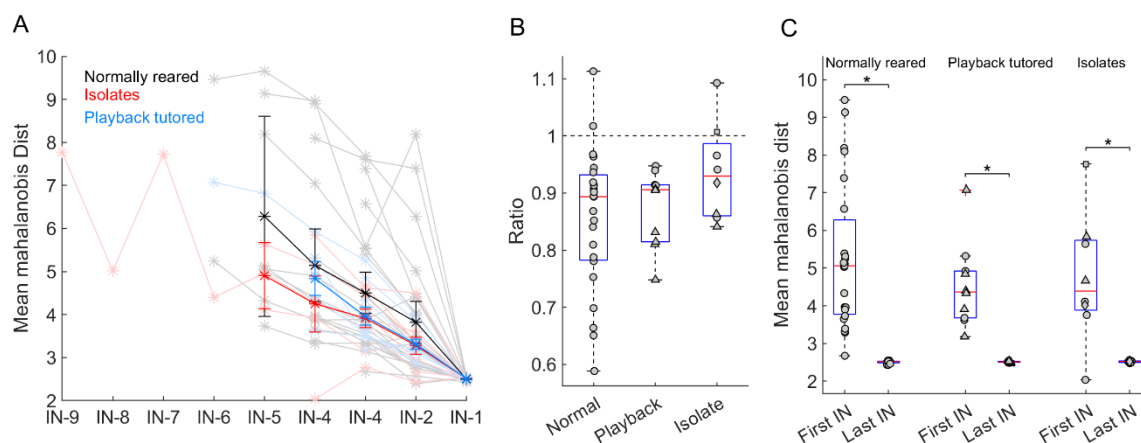


Figure 3.7 Acoustic progression is present in playback tutored birds and isolates.

A Mean Mahalanobis distance between the last IN and INs before. Dark lines indicate mean distance across birds; the light line represents individual birds. Black, red, and blue lines represent normally reared birds (n=23), isolates (n=8), and playback tutored birds (n=10). B. Box plot of mean ratio Mahalanobis distance between different INs for different groups. C. Box plot for mean Mahalanobis distance for first and last IN for all groups of birds. Each point represents a bird. Different shapes represent birds from different colonies. Circle: IISER, Pune, Triangle: McGill university, Square: Korea brain research institute, Diamond: Tufts university. Star represent p-value < 0.05, ANOVA (B), Wilcoxon signed rank.

3.4 Discussion

Different species of songbirds maintain the species-specific song pattern even when reared without exposure to the conspecific song (Lanyon E. Wesley, 1979; Price, 1979; Rice & Thompson, 1968). Here, I have used the zebra finch as a model system to compare the properties of introductory notes produced by birds reared with and without exposure to INs. Similar to previous studies, I have shown that birds reared without sensory exposure to INs begin songs with repetition of INs (Price, 1979). The mean number and structure of INs produced by isolates and playback tutored birds (tutored without INs) are similar to that of normally reared birds. Features like entropy, entropy variance, and pitch goodness differ from normally reared birds. While, duration, frequency modulation and mean frequency were similar. Further, inter-individual differences in the structure of INs produced by isolates and playback tutored birds are more than innate vocalizations like calls. Also, INs are structurally different from innate vocalizations like calls. The interval between successive INs becomes shorter and less variable for birds reared without sensory exposure to INs. Similarly, successive INs become more similar to the last IN, immediately before the song, in birds reared without sensory exposure to INs. Temporal similarity for INs and the motif is low for birds reared without sensory exposure

to INs compared to normally reared birds. Taken together, the temporal and acoustic progression of INs before the song was similar between normally reared, isolates, and playback tutored birds. Overall, these results suggest that birds reared without sensory exposure to INs produce INs with properties similar to that of normally reared birds. Further, these are structurally different from innate vocalizations like calls.

3.4.1 Similarities/differences in neural pathways involved in INs and song template

Normally reared birds are hypothesized to have a template consisting of INs and songs, which they use to produce a typical zebra finch song (Marler, 1990). Similar to the previous studies, my results, showing that isolate birds produce song bouts with INs followed by the song motif, suggests the presence of an innate template for INs and songs for isolates, too (Price, 1979). Birds tutored by playback of only song without INs also initiate song with INs. Here, birds are provided a template for songs but not INs. What happens in this case? Are the same regions involved in forming a template for INs as the motif syllable? One potential region could be NIf, which is known to send auditory input to HVC. A bilateral lesion in NIf (nucleus interfascialis of the nidopallium) (Roberts et al., 2012) or ablation of only HVC projecting NIf neurons before tutoring affects similarity to the tutor's song (Zhao et al., 2019). However, similar lesion after tutoring does not affect song learning, suggesting its role in forming memory of the tutor's song. In the same study, they have used optical stimulation to mimic tutoring. They show that stimulation of NIf projecting HVC neurons in male juvenile zebra finch during tutoring makes birds produce vocalization with a duration similar to stimulation following maturity. However, these birds also begin songs with INs (Zhao et al., 2019). One explanation for this could be that in case of the absence of a template for INs, HVC can form a stable sequence for INs, similar to song (Mackevicius et al., 2023), or NIf has a template for INs, or this information comes from a region other than NIf. It is likely that NIf doesn't store information regarding IN template, as birds that were optogenetically tutored, which is like imprinting a memory also initiate song with INs. However, it doesn't completely rule out role of NIf in template formation of INs, as birds tutored without exposure to INs also initiate song with INs. However, based on my previous result, in the presence of a template for INs, innate template can be overwritten. Furthermore, NIf also shows activity for the tutor's song, suggesting its role in forming the tutor's song template (Mackevicius et al., 2020). Does it also store information about the number and structure of INs the tutor produces? It would be interesting to see if the template for IN and song is stored in a different brain region or is

spatially distributed in the same brain region. In order to test if NIf is involved in storing template for INs as well, one can record neural activity during tutoring sessions and look for activity specific to IN number/ structure.

3.4.2 Neural pathway for IN production

Previous studies suggest different pathways are involved in the production of learned and innate vocalizations. Long call and song are two types of learned vocalizations produced by male zebra finches (Simpson & Vicario, 1990). Damage to the Ts nerve, which connects the brain to the syrinx and lesion in HVC (proper name), RA (robust nucleus of the arcopallium), and Uva (uvaformis), have been shown to affect the learned features of all vocalizations (Danish et al., 2017; Moll et al., 2023; Nottebohm et al., 1976; Williams & Vicario, 1993). However, RA lesioned birds do not sing but produce long calls, similar in structure to innate long calls produced by female zebra finches (Simpson & Vicario, 1990). Birds with unilateral Uva lesions occasionally produce a good song and recover their full songs within a few weeks after the lesion. However, it starts the song with degraded INs/syllables. Further, even after full song recovery, the structure of INs remains different from pre-lesion (Coleman & Vu, 2005). Uva is active before calls and INs (Danish et al., 2017; Moll et al., 2023; Williams & Vicario, 1993). Further, similar to the song, INs have learned and innate properties. Overall, these results suggest that the mechanism of production of INs could be similar to other learned vocalization-like songs.

3.4.3 Temporal and acoustic progression

Birds reared without exposure to INs produce INs with characteristic temporal and acoustic progression similar to normally reared birds, suggesting these two properties are innate. Zebra finches cross-fostered with bengalese finches learn the syllables of a tutor. However, the temporal pattern is similar to normally reared zebra finches, suggesting a biological predisposition to produce certain temporal pattern (Araki et al., 2016; James et al., 2023). These could be due to peripheral or central constraints to produce a song with a tempo similar to the zebra finch. Here, peripheral and central constraints refer to the respiratory and neural constraints respectively. Birds produce vocalization during the expiratory phase of the respiration. In order to meet their respiratory requirements while singing, birds take deep inhalations, which are called mini-breaths (Calder, 1970). These mini-breaths help birds to replace the air used up during vocalization. Birds take shorter mini-breaths following a short

vocalization. It has been shown that canaries can sing longer songs when accompanied by mini-breaths (Hartley & Suthers, 1989; reviewed in Suthers, 2001). Another example that suggests the influence of peripheral constraints on song tempo is the song produced by the song sparrow tutored with a species-specific song at a higher repetition rate. The pupil produces a song with abnormally broken syntax; that is, adds pauses within the song (Podos, 1996). Overall, this sets a limit for the rate at which a bird can produce vocalizations. The central constraints in song production can arise due to the inability of the sensory system to respond to a particular stimulus, thus limiting the learning capabilities. A previous study has shown that an auditory brain region of a male zebra finch called Field L shows neural activity in response to playback of noise with inter-syllable intervals within the zebra finch song range (Araki et al., 2016). Apart from this, zebra finches show a biological predisposition to learning intervals within species-specific song range (James et al., 2023). This suggests that birds have sensory bias for the species-specific song. Apart from the sensory bias, species specificity can also arise due to neural circuitry or properties of specific neurons. In drosophila species, it has been shown that a homologous descending neuron codes for different song types in similar social contexts (Ding et al., 2019). In the case of songbirds, namely the zebra finch and its closely related species, the owl finch, it has been shown that certain transcription factors are differently expressed in brain regions controlling song production (RA). BDNF is one of the trans-regulated gene whose expression level showed species-specific difference. Further, the overactivation of the BDNF receptor in RA affected features like the inter-syllable gap of adult zebra finch song (Wang et al., 2019). Recent study suggested the role in genetic component in bias toward learning specific song (Toji et al., 2024). Thus, overall, the changes at the neural level, which I have referred to as central constraint, might be responsible for the species-specific song features.

Thus, even if number and structure are learned from the tutor, the repetition and temporal/acoustic progression of INs are innate. Rajan & Doupe (2013) have suggested that INs be involved in preparing the bird for the upcoming song. They have shown that AreaX projecting HVC neurons fire differently for the last IN (IN immediately before the first motif syllable) (Rajan & Doupe, 2013). If temporal progression in INs is involved in the preparation of an upcoming song, following stimulation in HVC during INs and/or the interval between INs, one could expect a delay in the song's onset or slight changes in the song. This would be similar to stimulation during the preparatory phase for cued movements, where stimulation during preparatory phase delays movement onset (Churchland & Shenoy, 2007). Thus, temporal progression might be a readout of motor preparation. At the same time, the acoustic

progression might give an idea about the repeat number. Previous study has shown that the acoustic feature of the upcoming syllable is influenced by the preceding and following syllable (Wohlgemuth et al., 2010); acoustic progression might reflect the position of IN in the sequence and not necessarily represent preparation.

3.4.4 Learned features of INs

Even though both isolates and playback tutored birds begin the song with INs (Kalra et al., 2021; Chapter 2), I found differences in the acoustic structure of these INs relative to INs produced by normally reared birds. Birds reared without exposure to INs had lower entropy, pitch goodness, and higher entropy variance than normally reared birds. Birds reared in isolation produce song syllables with higher entropy, suggesting entropy is a learned feature for song and IN (Zhao et al., 2019). This suggests that these properties are influenced by learning. Further, INs produced by isolates and playback tutored birds differ from other innate calls produced, supporting that they are similar to other learned vocalizations. Learning of these features by the tutor/father might provide nest-specific identity to the juvenile, while the innate acoustic features might provide species-specific identity.

3.4.5 Species-specific characteristics

Many species of oscine birds initiate songs with INs (Ota & Gahr, 2022; Richards, 1981; Soma & Iwama, 2017; Sossinka & Böhner, 1980). Different birds produce different types of INs; for instance, the java finch (Soma & Iwama, 2017), like the zebra finch, begins the song by repeating a syllable (Price, 1979; Sossinka & Böhner, 1980; Zann, 1996), while the white crown sparrow initiates the song with a characteristic whistle (Soha & Marler, 2000). This suggests there could be a biological predisposition to produce INs before song for different species; the type of INs produced might differ. Here, I have shown that zebra finches reared without exposure to INs also start the song with INs. The INs these birds produce have temporal properties similar to normally reared birds, suggesting it is innate. Interestingly, suboscine birds (birds that do not learn song) also show temporal progression in INs (Chelén et al., 2005; Kroodsma et al., 1987). However, all birds might not have temporal progression, as some songbird species, like white crown sparrow, have only one introductory whistle (Soha &

Marler, 2000) unlike the zebra finch (Price, 1979; Sossinka & Böhner, 1980; Zann, 1996), java finch (Soma & Iwama, 2017), which have repeating syllables as INs. It suggests that INs might be present across different bird species, but the temporal pattern could depend on the species. The presence of common features across birds might suggest its importance in producing a subsequent song or a common underlying neural mechanism to start a song. This suggests that temporal progression could be a common property of INs across many species of birds. It would be interesting to compare the properties of INs across species. At the same time, differences will give insight into its function, like species identification. However, further studies will be required to confirm either.

Overall, similar to the previous study, I have shown that INs have both learned and innate component. Zebra finches have innate tendency to start song with repetition of INs consisting of temporal and acoustic progression. These innate properties of INs might provide as species-specificity, while learned features give nest specific identify.

3.5 References

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Chapter 4

*Brain region involved in the
learning of introductory notes*

4.1 Introduction

Many animals start their communicative display with introductory gestures. Among these, the zebra finch, a well-studied Australian songbird (Zann, 1996), initiates its song with introductory gestures called introductory notes (INs) (Price, 1979; Sossinka & Böhner, 1980; Zann, 1996). Introductory notes are short-duration repetitive vocalizations that precede a consistent song (also called the motif). Zebra finch learn their song from an adult male, typically their father (Immelmann Klaus, 1969; reviewed in Marler, 1990). Previously, I have shown that INs similar to song have both learned and innate components (Kalra et al., 2021; Chapter 2, Chapter 3). Given that INs are also vocalization similar to the song, it is interesting to look whether similar brain regions control the learning of both song and INs. Here, I have checked if the brain region responsible for song learning also influences the learning of INs in zebra finches. Song learning in zebra finches typically takes place within the first 90 days post-hatching, following which song does not change. This song learning consists of two phases, namely, (1) the sensory acquisition phase from ~ 20 days till ~ 60 days post-hatching and (2) the sensory motor phase from ~35 days till ~90 days post-hatching. During the sensory acquisition phase, juveniles form a memory of the tutor's song, and in the sensory motor phase, they practice and

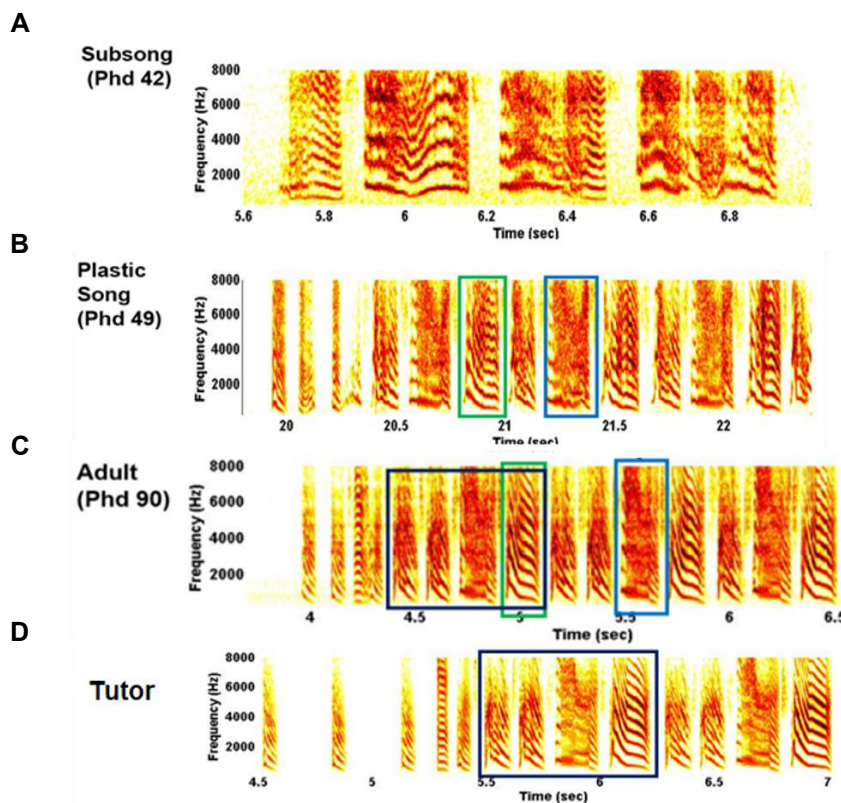


Figure 4.1 Spectrogram of song produced by male zebra finch during the song learning phase

A. Subsong produced by bird around 42 days post-hatch. It consists of unrecognizable syllables. B. Spectrogram of plastic song produced by the same around 49 days post-hatch. Green and blue boxes highlight recognizable syllables found at day 90 as well. C. Spectrogram of song produced by the same bird at day 90. D. Spectrogram of the tutor's song. Dark blue rectangle represents motif that is similar between tutor and pupil.

refine their vocalizations to become similar to their father's (or tutor's) song. Following this period, the song doesn't change and is usually similar to the father's (or tutor's) song.

The song produced during this song learning phase is categorized into three stages: (1) subsong (consists of unrecognizable vocalization) (Figure 4.1A), (2) plastic song (consists of few recognizable vocalizations) (Figure 4.1B) and (3) the final crystallized song (Figure 4.1C) (Immelmann Klaus, 1969; Marler, 1990). Different brain regions are involved in song learning. Two major brain pathways have been identified to be involved in song production and learning in zebra finches. One pathway involved in song production is called the motor pathway (Nottebohm et al., 1976), and the other pathway required for song learning and plasticity is called the anterior forebrain pathway (Bottjer et al., 1984; Brainard & Doupe, 2000; Scharff & Nottebohm, 1991; Williams & Mehta, 1999) (Figure 4.2). The motor pathway consists of a premotor nucleus called HVC (proper name) and the motor nuclei called RA (robust nucleus of the arcopallium). HVC projects to RA, and RA further projects to the tracheosyringeal portion of the hypoglossal nucleus (nXIIts) and the brain stem respiratory centers (RAM (nucleus retroabigualis) and PAM (nucleus paraambigualis). The nXIIts project to the syringeal muscles, thereby controlling vocalization (Nottebohm et al., 1976; Wild, 1993). Overall, both the syringeal muscles and respiratory center are coordinated to produce vocalization during singing. The anterior forebrain pathway (AFP) consists of AreaX, DLM (dorsolateral nucleus of the medial thalamus), and LMAN (the lateral portion of the magnocellular nucleus of the anterior nidopallium). AreaX (basal ganglia homolog in humans) receives projections from the cortical area called HVC (Nottebohm et al., 1976), and it further projects to DLM (thalamic nuclei). DLM sends its projection to LMAN. LMAN projects to RA and send projections back

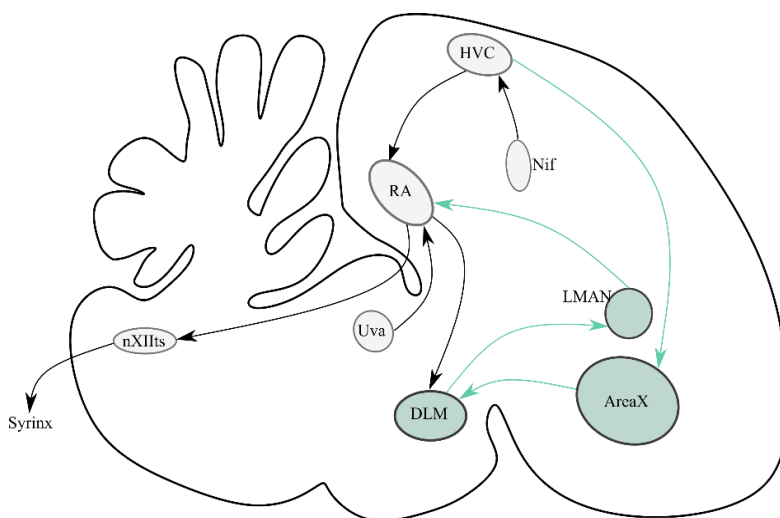


Figure 4.2 Sagittal section of male zebra finch brain highlighting different brain regions involved in song learning and production. HVC (proper name), RA (robust nucleus of the arcopallium), DLM (dorsolateral nucleus of the medial thalamus), LMAN (the lateral portion of the magnocellular nucleus of the anterior nidopallium), Nif (nucleus interfascicularis of the nidopallium), Uva (Uvaeformis), nXIIts (tracheosyringeal portion of the hypoglossal nucleus). Green highlights anterior forebrain pathway which is involved in song learning.

to AreaX (Bottjer et al., 1989; Vates & Nottebohm, 1995). This forms the cortical basal ganglia thalamus loop similar to humans (reviewed in Fee & Scharff, 2010). Lesioning of LMAN (the output of the anterior forebrain pathway) in male zebra finches during the song learning phase (plastic) affects the accuracy of song copying from the tutor.

Following the lesion, the song becomes stereotyped, consisting of syllables it had already learned. Lesioning LMAN after birds have learned the tutor's song does not affect the song (Bottjer et al., 1984; Scharff & Nottebohm, 1991). Overall, LMAN, which forms part of the anterior forebrain pathway, affects the accuracy of song copying in the male zebra finch. In Chapter 2 and Chapter 3, I have shown that INs, similar to songs, have both learned and innate components. The learned properties of INs include the number and structure (Kalra et al., 2021). While, the innate components are temporal and acoustic progression to a “consistent” state before motif onset (Chapter 3). If INs are learned the same way as songs, one would expect brain regions that affect song learning to influence the learning of INs. In order to check if LMAN (and the AFP) is involved in learning INs as well, I have performed bilateral electrolytic lesions of LMAN in juvenile male zebra finches. Birds with 20-60% LMAN lesion showed lower similarity in the structure of INs with the tutor than birds with less than 20%. In contrast, the number of INs produced by both groups of birds is similar to the tutor. However, due to the fewer birds with LMAN lesions, it is difficult to conclude if learning of IN number and structure is affected by LMAN lesion.

4.2 Materials and Methods

I have analyzed songs from birds that were recorded at different locations (n= 7, IISER Pune; n=1, Tufts University, Boston; n=3, UCSF and n=4, McGill University, Canada). For all birds recorded at IISER Pune, all procedures at IISER Pune were conducted after approval from the Institute Animal Ethics Committee (IAEC), IISER Pune, and were in accordance with CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), New Delhi. For all birds recorded at McGill University, all procedures at McGill were conducted following approval from the McGill University Animal Care and Use Committee in accordance with the guidelines of the Canadian Council on Animal Care. For all birds recorded at Tufts, all procedures at Tufts University were approved by the Tufts University Institutional Animal Care and Use Committee. For birds recorded at UCSF, all procedures performed at UCSF were

in accordance with NIH guidelines and were approved by the UCSF Institutional Animal Care and Use Committee.

4.2.1 Experimental birds

Lab bred male zebra finches were used for LMAN lesion experiments. 36 male juvenile zebra finches, 2 female juvenile zebra finches, 14 adult male zebra finches were used in this study. LMAN lesions were performed on 29 male juvenile birds; antidromic stimulation in RA was tried in 7 juvenile and 14 adult birds to identify LMAN. Birds used for breeding/tutors were either lab bred or bought from outside. Data for socially tutored birds is the same as in Chapter 2. Isolate bird data is the same as that used in Chapter 3.

4.2.2 Experimental design

Juvenile zebra finches were socially tutored for this experiment to cover a wide range of IN number across tutors. In order to test if juveniles copy mean IN number for the tutor, I wanted to use birds with extreme INs as the tutor. Previously, I had shown that birds reared in absence of tutor, tend to produce a mean of 3 IN (Kalra et al., 2021). Thus, in order be sure that birds actually copied from the tutor, I decided to use birds that produced either low or high mean IN. Father was separated from the nest when birds around 10 days post-hatch (range: 11-13 days post-hatch), except for 1 nest with 3 hatchlings, where the female had died (birds were reared with biological father). Juveniles were raised with their mother and siblings till 35 days post-hatch, after which they were kept with a tutor (range: 35-43 days hatch, median: 37). Juveniles were kept with a tutor till 90 days post-hatch (range: 85-103 days post-hatch, median: 94; hatchlings of nest that was kept with father was separated on 153 days post-hatch). Juveniles

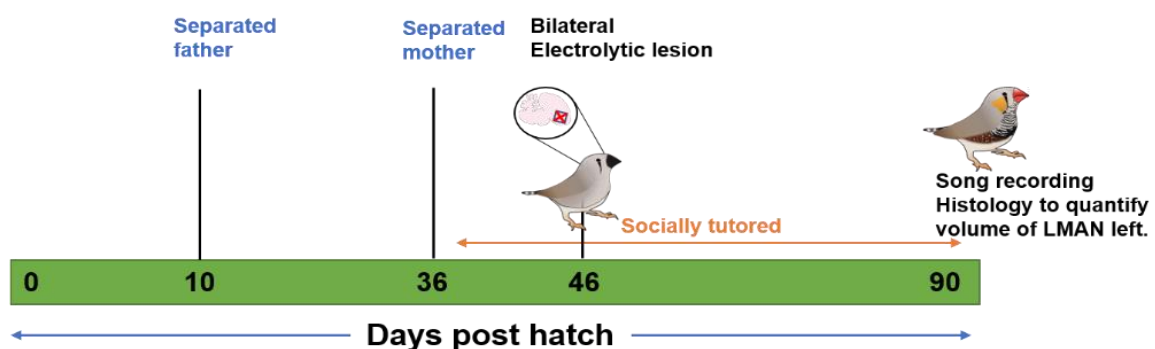


Figure4.3 Schematic of the experimental design.

were separated with cardboard partitions from other birds in the colony and could visually see

their tutor. Juveniles could hear other birds in the colony. The bilateral electrolytic lesion of LMAN was done when birds were around 46 days post-hatch (range: 44-54 days post-hatch, median: 49). Birds were recorded for 2 days before and after the lesion and in adulthood (> 90 days post-hatch, range: 91-172, days post-hatch, median: 99). Birds were euthanized (range for age at perfusion: 204-1017 days post-hatch, median: 595, 3 birds had died before being perfused (range: 326-564), but the brain was extracted following death for histology) to determine the extent of lesion following the final recording.

4.2.3 Surgery

All surgeries were performed under anesthesia. Birds were given meloxicam as an analgesic (Dosage: 0.25mg/kg) 1 to 1.5 hours before surgery. Birds were anesthetized either using injectables (n=4) (ketamine (30 mg/kg), xylazine (3 mg/kg) and diazepam (7 mg/kg) given intramuscularly) or isoflurane (flow rate: 1ltr/min, concentration was maintained between 2-4%) (n=27). Antidromic stimulation was done under urethane (n = 9) or isoflurane (n=12). Since birds cannot regulate their temperature under anesthesia, a customized bird jacket was put on the bird and a heat lamp was placed near the bird throughout the surgery. Before being placed on the stereotaxic instrument (Narshige, custom-designed), ear feathers were cut to expose the ear. To prevent movement of the head during surgery, the head was held between ear bars, and the beak was placed in the beak bar of the stereotaxic instrument. Lignocaine, a local anesthetic drug (~25 ul), was injected subcutaneously, and the skin was cut open to expose the skull. The first layer of the skull was removed over 'y' (major blood vessel, used for stereotaxic coordinates) for clear visualization. An electrode was then aligned to the center of the branch point of the 'y' and carefully moved to the LMAN coordinates (Anterior – posterior (AP): 4.2-4.9 mm, medial-lateral (ML): 1.5-1.9 mm; obtained from a stereotaxic atlas (<https://www.ncbi.nlm.nih.gov/books/NBK2348/>)). The electrode was lowered, and current was passed at two depths, 2.1 mm, and 2.4 mm, for lesioning. Lesions were done using a nichrome wire tetrode (n = 21) or tungsten electrode (n=1). Lesions were performed with negative polarity current (50,100,150 μ Amp for 100 secs). Following the lesion, bone wax and dental cement were used to close the exposed brain. Antidromic stimulation was done by implanting a stimulating electrode in RA and a recording electrode in expected LMAN coordinates based on the atlas. A monophasic pulse of 200 μ secs (current range 100-300 μ Amp) in duration was used for stimulation. However, no antidromic spike was observed.

Table 4.1 Information regarding LMAN coordinates, current etc for LMAN lesion

S.No	Bird name	Days post-hatch lesion	AP (mm)	ML (mm)	Depth (mm)	Duration; secs (Current; μ Amp)	Type of electrode used	comments
1	yellow87	49 - 50	4.2, 4.5, 4.9	1.5, 1.8	2.5	100 (100)	Tetrode	Survived
2	red031	47 - 49	4.5,4.7,4.9	1.6, 1.9	2	100 (100)	Tetrode	Survived
3	white64	45 - 48	4.5, 4.7, 4.9	1.6,1.8	2.4	100 (100)	Tetrode	Survived
4	white65	46 - 49	4.5, 4.7, 4.9	1.5, 1.8	2.4	100 (100)	Tetrode	Survived
5	white63	47 - 50	4.5,4.7,4.9	1.5, 1.8	2.4	100 (100)	Tetrode	Survived
6	black53	46 - 47	5.3, 5.5, 5.7	1.5, 1.8	2.3	100 (100)	Tetrode	Survived
7	pink007	48 - 49	4.5, 4.8, 5.1	1.5, 1.8	2, 2.4	100 (100)	Tetrode	Survived
8	pink031	48 - 49	4.5, 4.8, 5.1	1.5, 1.8	2, 2.4	100 (100)	Tetrode	Survived
9	pink032	45 - 49	4.8, 5, 5.2	1.6, 1.9	2.1, 2.4	100 (100)	Tetrode	Survived
10	pink033	48 - 52	4.8, 5, 5.2	1.6, 1.9	2.1, 2.4	150 (150)	Tetrode	Survived
11	pink034	49 - 53	4.8, 5, 5.2	1.6, 1.9	2.1, 2.4	150 (150)	Tetrode	Survived
12	pink010	45 - 49	4.8, 5, 5.2	1.6, 1.9	2.1, 2.4	150 (150)	Tetrode	Survived
13	pink011	47 - 51	4.8, 5, 5.2	1.6, 1.9	2.1, 2.4	150 (150)	Tetrode	Survived
14	pink012	50 - 54	4.8, 5, 5.2	1.6, 1.9	2.1, 2.4	150 (150)	Tetrode	Survived
15	pink018	45 - 48	4.8, 5, 5.2	1.6, 1.9	2.1, 2.4	150 (150)	Tetrode	Survived
16	pink093	45 - 47	4.8, 5, 5.2	1.6, 1.9	2.1, 2.4	50 (100)	Tungsten	Survived
17	orange55	47	-	-	-	-	-	Died during surgery
18	red032	46-48	4.2, 4.5, 4.9	1.5, 1.8	2, 2.4	100 (100)	Tetrode	Died after 2 days
19	black54	49-50	-	-	-	-	-	Died during surgery
20	red052	43-44	4.8, 5, 5.2	1.6, 1.9	2.1, 2.4	100 (100)	Tetrode	Died next day
21	pink035	48	-	-	-	-	-	Died during surgery
22	pink022	47-48	-	-	-	-	-	Died during surgery
23	pink023	46-47	4.5, 4.8, 5, 5.2	1.6, 1.9	2.1, 2.4	150 (150)	Tetrode	Died next day
24	pink026	47-49	4.5, 4.8, 5, 5.2	1.6, 1.9	2.1, 2.4	150 (150)	Tetrode	Died after 2 days
25	pink024	51-53	-	-	-	-	-	Died during surgery
26	pink025	48-50	4.5, 4.8, 5, 5.2	1.6, 1.9	2.1, 2.4	150 (150)	-	Died after 2 days
27	pink052	45-46	-	-	-	-	-	Died during surgery
28	pink053	47-48	4.8, 5, 5.2	1.6, 1.9	2.1, 2.4	100 (100)	Tetrode	Died next day
29	pink051	48-49	4.8, 5, 5.2	1.6, 1.9	2.1, 2.4	100 (100)	Tetrode	Died after 2 days

4.2.4 Visualization of lesion

Birds were perfused, and brains were fixed using 4% paraformaldehyde (PFA) (made in 1X PBS). Brains were kept for two days in PFA, then transferred to sucrose (30% in PBS) for a day. The brain was sectioned (sagittal) using a cryostat (Cryostar NX70 cryostat) to get 40 μ m thick sections. Sections were initially collected in 24 well plates containing 1X PBS. Following this area of LMAN left was visualized as described below.

4.2.4.1 Immunohistochemistry

I first used immunohistochemistry (IHC) to visualize the extent of LMAN lesion. A subset of sections were taken in a 24-well plate for staining. Sections were initially fixed in 4% paraformaldehyde (PFA) for 15 mins. Following this, 10 mins washes were done in Phosphate buffer triton X-100 (PBT; 0.3% made in 0.02M Phosphate buffer) on a shaker for permeabilization, and the process was repeated 3 times. Next, sections were kept in 0.5% H₂O₂ for 10 mins in the dark to quench endogenous peroxidase, followed by a single 5 mins wash in PBT on the shaker. Blocking was done using normal goat serum (5% in 0.3% PBT for 1.5 hours on the shaker). Sectioned were incubated in primary antibody (CGRP, Sigma C7113/ PV, Swant 235/ PARV, Sigma P3088/ anti GABA, Sigma A0310) for 4 hrs. at room temperature and then kept at 4° Celsius overnight. I have tried different primary antibody dilutions (range 1:1000 to 1:10,000) in 0.3% PBT and 2% goat serum. The following day, 10 mins washes were given in 0.3% PBT, which was repeated 3 times. These sections were then incubated at room temperature for 1 hr. in 1:1000 dilution of peroxidase conjugated secondary antibody (Sigma A9044, Anti-mouse IgG (whole molecule)). To remove excess antibody, 10 mins washes were given in 0.3% PBT on a shaker and was repeated four times. 3,3' diaminobenzidine (DAB) reaction was then used for visualization. DAB solution was made by dissolving 1 tablet DAB and Urea Hydrogen Peroxide (Sigma, D4293) in 4.5 ml of Milli-Q water along with 0.5 ml of 0.3% NiCl₂ (enhances contrast). Color was allowed to develop for 10 mins. In cases where color didn't develop in 10 mins, it was kept longer. To stop the reaction, sections were washed with Milli-Q water. Following this, sections were given, 10 mins washes in 0.02M PB thrice. Following this the sections were stored in 0.02M PB. On next day sections were mounted on poly-L-Lysine solution (sigma, P8920-100ml) coated slides (1:10, dilution of Poly L Lysine in Milli-Q was used for coating slide.). These were then air dried for 2.5 hrs., kept for 1 minute in 70% ethanol, then transferred

to 100% ethanol (twice, 1 minute each), and then kept for 1 min in xylene. Following this, DPX (mounting media) was spread on the slide, and the coverslip was placed carefully, preventing air bubbles. Excess DPX was removed the following day, slides/coverslips were cleaned with 70% ethanol, and sections were visualized under a bright field microscope (Olympus MVX10) at 0.8x magnification.

Following standardization, I could get specific staining for HVC and RA clearly with PV (Swant 235), but LMAN was very lightly stained (Figure 4.4A, B). Furthermore, with CGRP (Sigma, C7113) very faint staining was observed for LMAN and RA (Figure 4.4C). In both



Figure 4.4 Immunohistochemistry of sagittal sections of zebra finch brain.

IHC done using PV (A,B), 1:1000 dilution was used and CGRP (C) 1:5000 dilution was used.

cases it was difficult to mark the boundaries, therefore, I didn't continue with IHC.

4.2.4.2 Cresyl violet

To perform cresyl violet staining, 40 μ m sagittal brain sections were mounted on a poly L Lysine-coated slide a day before staining. I had tried two different protocols for cresyl violet staining. In order to check if different brain regions could be clearly differentiated from the background, a control set of slides was always kept for staining with the LMAN lesion brain sections. This was to assure that the absence of LMAN was not due to improper staining, rather it was been damaged.

- **Procedure 1**

Slides were kept in different concentration of ethanol: 100 % for 5 mins, then 70% ethanol for 2-3 mins, then 20% ethanol for 2-3 mins. This was followed by a 1-2 mins wash in Milli-Q water. Following this, slides were dipped in cresyl violet solution (0.5%) for 5-10 minutes with regular check at staining. Then, 2 dips were given in Milli-Q water, 2 dips in differentiator – I (consisting of 270 ml of 70% ethanol and 3 ml acetic acid (100%)), 1-2 dips in differentiator – II (consisting of 270 ml of 100% ethanol and 3 ml of acetic acid

(100%)) and 1-2 dips in 100% ethanol. This was followed by a 5 minutes dip in xylene. Sections were then cover slipped after adding mounting media DPX.

This method didn't give reliable staining each time it was used. Hence, I tried the following procedure for cresyl violet staining.

- *Procedure II*

Slides were dipped in 1% cresyl violet (cresyl violet acetate, sigma, C5042-10G, prepared by dissolving 1g cresyl violet acetate in 100 ml Milli-Q with 0.136g sodium acetate and 0.539 ml of 100% acetic acid. This was kept with stirring overnight in the dark and filtered next day with Whatman filter # 1 in dark) for 10 mins, then 2-3 dips were given in Milli-Q water. Then, slides were dipped in 50%, 50%, 70%, and 70% ethanol for 1 min each. After this, slides were kept in 95% ethanol for 5 minutes and checked for staining at regular intervals. In the control set of sections (brain section with no LMAN lesion), the differentiation of different nuclei like HVC, RA, AreaX and LMAN from the background was used as a marker for good staining. This was followed by 2 fast dips in 100% ethanol and 3 dips in xylene. Sections were then cover slipped after adding mounting media DPX. Excess DPX was removed the next day, and slides/coverslips were cleaned with 70% ethanol and visualized under a bright field microscope (Olympus MVX10) at 0.8x magnification (Figure 4.5).

Following either procedure, I didn't get reliable staining each time the procedure was repeated. Hence, I was not able to use cresyl violet staining for visualization of LMAN.

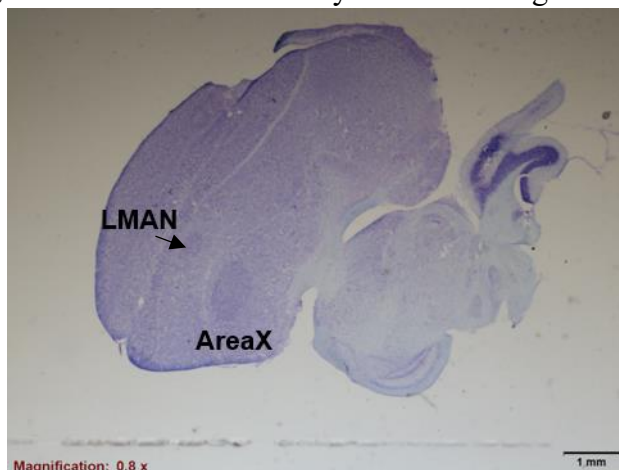


Figure 4.5 Cresyl violet stained sagittal sections of zebra finch brain.

4.2.4.3 Visualization of unstained sections

40µm sagittal brain sections were directly mounted on poly-L-Lysine coated slides. Sections were kept for 5 min to air dry (this is to allow sections to stick to the slide); when sections were still partly wet, slides were dipped in xylene for 1 min. DPX was applied to the slides, and the cover slip was carefully placed without air bubbles. On the following day, excess DPX was removed, and slides/coverlips were cleaned with 70% ethanol and visualized under a bright field microscope (Olympus MVX10).

Overall, IHC and cresyl violet didn't give reliable staining, hence, I didn't use them for the visualization of LMAN. I visualized the extend of LMAN remaining using unstained sections.

4.2.5 Song recording

Undirected songs (produced in absence of female zebra finch) of male birds were recorded two days before surgery, two days after surgery, and in adulthood (around 90 days post-hatch). Before surgery, birds were recorded along with the tutor, and following surgery, juveniles were recorded alone. Songs were recorded in an acoustic chamber (NewTech Engineering Systems, Bangalore, India) using a microphone (AKG 517) placed above the bird's cage. A custom-written Python script was used for song recording. Birds were recorded using a continuous mode of data acquisition and data was written to disk at a sampling rate of 44100 Hz. See Chapters 2 and 3 methods for details regarding song recording of socially tutored and isolate birds.

4.2.6 Data analysis

Data analysis was done using custom-written scripts in MATLAB (MathWorks). The panel function was used to generate the final figures (Panel - File Exchange - MATLAB Central). ImageJ was used for calculating the area of LMAN left after lesioning.

4.2.6.1 Classification of a song bout

Vocalizations were separated into bouts and classified into motif syllables, INs and calls as described in Chapter 2 (see Methods Chapter 2).

4.2.6.2 Segmentation and syllable annotation

Segmentation and annotation procedure similar to Chapter 2 was followed (see Methods, Chapter 2).

4.2.6.3 Counting of INs

Counting INs is similar to previous chapters (see Methods Chapter 2).

4.2.6.4 Temporal progression and acoustic progression of INs

Temporal and acoustic progression was calculated in same way as mentioned in Chapter 3 (see Methods, Chapter 3).

4.2.6.5 Similarity

Sound Analysis Pro was used for calculating similarity (<http://soundanalysispro.com/>) (Tchernichovski et al., 2000). An asymmetric time course was used for calculating motif similarity, and a Symmetric time course was used for INs. In order to calculate the pre-lesion similarity, a stretch of 1 sec from the beginning of the song bout from the juvenile recording was used. Default setting were used for calculating song similarity. For IN similarity all setting were default except min dur, which was reduced to 3 msec.

4.2.6 Statistics

Pearson correlation coefficient was calculated using MATLAB function `corrcoef` wherever necessary.

4.3 Results

4.3.1 LMAN might play a role in learning IN structure, but not number during the song learning phase.

Previously, we have shown that the male zebra finch learns the number and structure of INs from the tutor, like the song syllables. As mentioned before, LMAN is involved in song learning. In order to check if LMAN is also involved in learning INs, I performed bilateral electrolytic lesions of LMAN in male juvenile zebra finches during the song-learning phase (see Methods). I tried multiple staining techniques to quantify the volume of LMAN left, but LMAN was not

consistently visible with any of these techniques (see Methods). So, I used wet, unstained, sagittal brain sections that were cover slipped and imaged using a bright field microscope. ImageJ software was used to mark the LMAN border and quantify its area. Based on the volume of LMAN left, birds were classified into three categories: birds with <20% lesion (n=6), birds with 20-60% lesion (n=7), and birds for which I could not measure the area of LMAN remaining (n=3). Sagittal brain sections of the unlesioned brain that were used as a control are shown in Figure 4.6 (Yellow circle/oval marks LMAN). Images of the brain section with marked LMAN for a bird with <20% lesion and 20-60% lesion are shown in the appendix (Appendix Figure A3 (<20% lesion), A4 (20-60% lesion)).

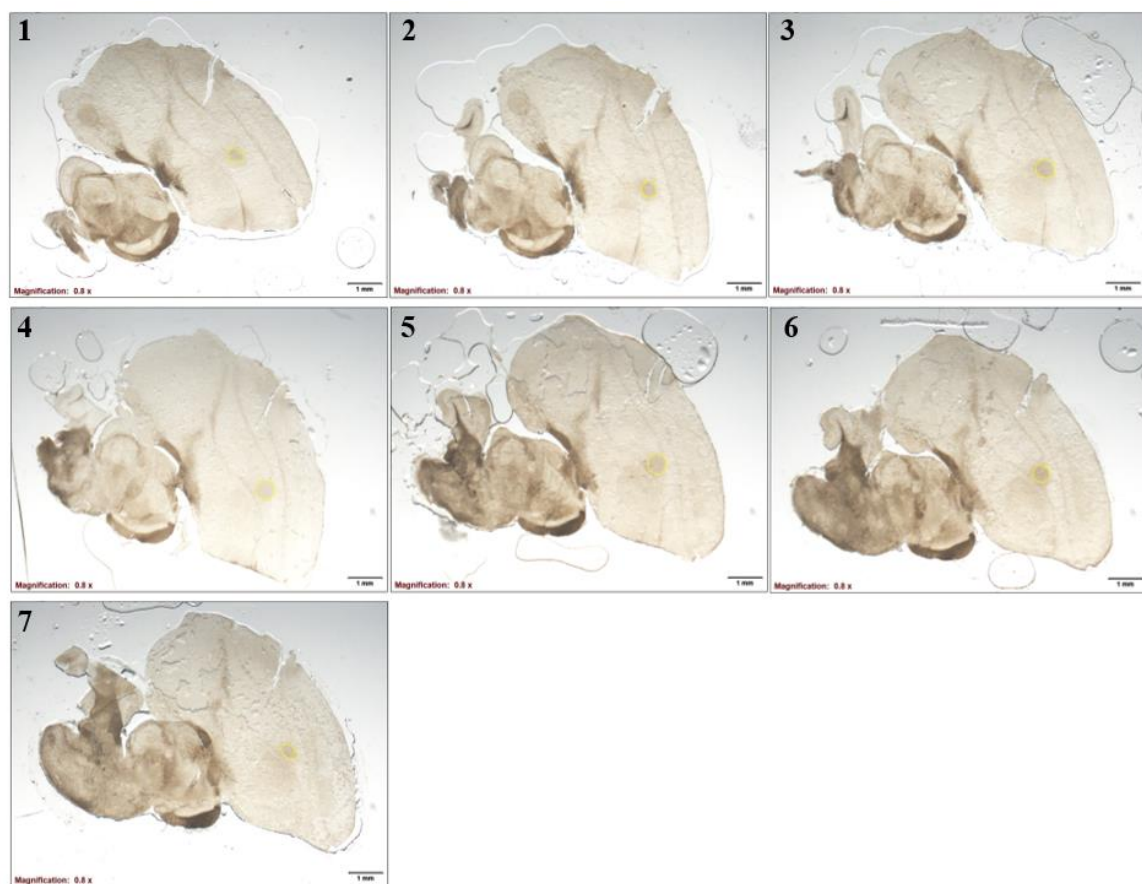


Figure 4.4. Sagittal sections of zebra finch brain visualized using bright field microscope for a control bird

Yellow polygon in all sections marks LMAN and the area of this polygon was calculated using imagej.

Number on the top left corner shows section number.

Sound Analysis Pro (SAP) was used to calculate the motif and IN structural similarity. Birds with <20% LMAN lesion had the least similarity with the tutor's motif compared to birds with 20-40%

lesion, undetermined and socially tutored birds (Figure 4.7A). Further, I calculated IN similarity between tutor and pupil using SAP to check if IN structural similarity is affected by LMAN lesion. Compared to other birds, IN structural similarity was lesser for birds with 20-60% lesion (Figure 4.7B). Next, I compared the mean number of IN between tutor and pupil for all groups of birds. Unlike socially tutored birds, there was no correlation between tutor and pupil mean IN number for birds with lesions (Figure 4.7C, 4.5D, 4.5E). However, there seems to be a trend: birds tutored with low IN tutors have a low mean IN number, and birds tutored with high IN tutors have a high mean IN number. Overall, this suggests that learning IN structure, but not the number of INs in the juvenile male zebra finch, might involve LMAN. However, due to a smaller number of birds with lesion, it is difficult to conclude the involvement of LMAN in learning of INs.

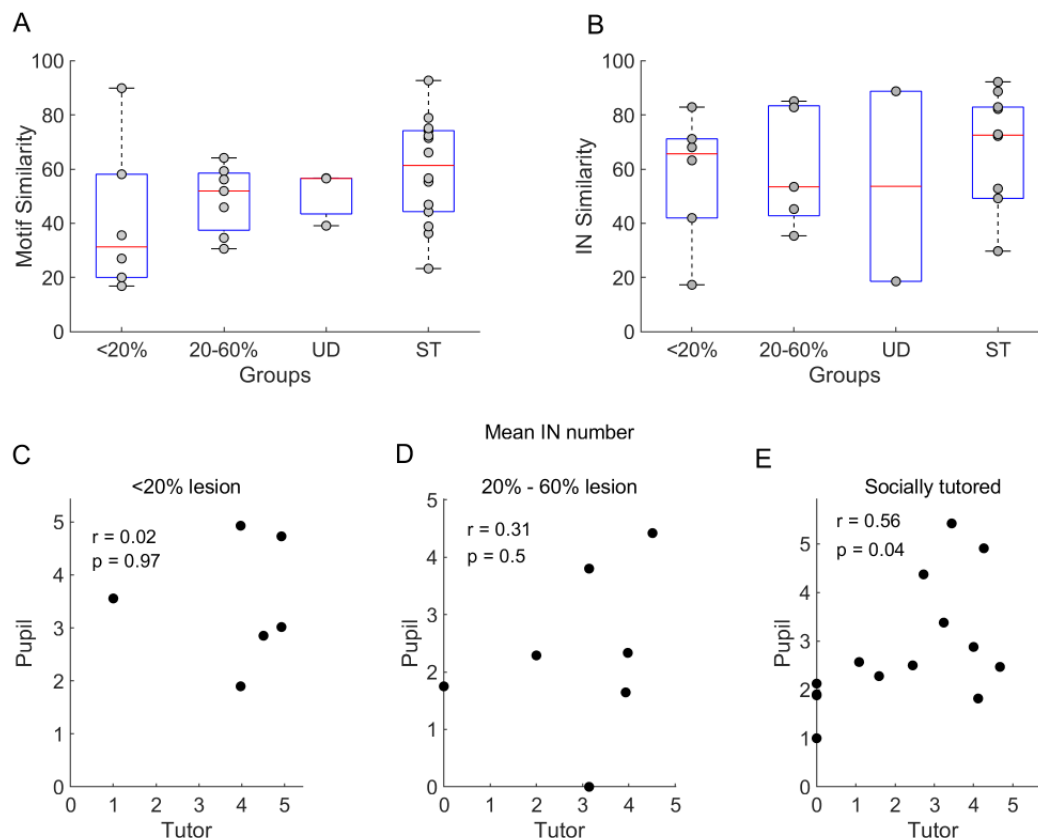


Figure 4.7 Learning of IN structure, but not number might involve LMAN

Box plot of similarity between tutor and pupil motif (A) and IN (B) for different categories of birds namely, <20% lesion (n=6), 20-60% lesion (n=7), undetermined (UD) (n=3) and socially tutored birds (ST) (n=14). Number in bracket show number birds for motif similarity. Number of birds for IN similarity are, <20% lesion (n=6), 20-60% lesion (n=5), UD (n=2) and ST (n=10). Mean IN number of tutor vs pupil for birds with <20% lesion (C), 20-60% lesion (D), socially tutored birds (E). Each circle represents a birds. Pearson correlation coefficient was calculated.

4.3.2 Learning of INs and motifs is not equally affected by LMAN.

I have shown that learning IN structure and number is independent of learning (Figure 2.10, 2.11, Chapter 2; Kalra et al., 2021). Further, to check if the LMAN lesion affects the learning of INs and motifs equally, I plotted motif similarity vs. IN structure and number similarity with the tutor (Figure 4.8A, B). There was no correlation between either, suggesting that IN is learned independently of the song. Further, this did not depend on the amount of LMAN lesion. This suggests that LMAN does not equally influence the learning of IN and song. Also, birds with <20% lesion have lower song similarity to tutor but have higher IN similarity. Furthermore, there is no correlation between IN similarity and difference in mean IN number

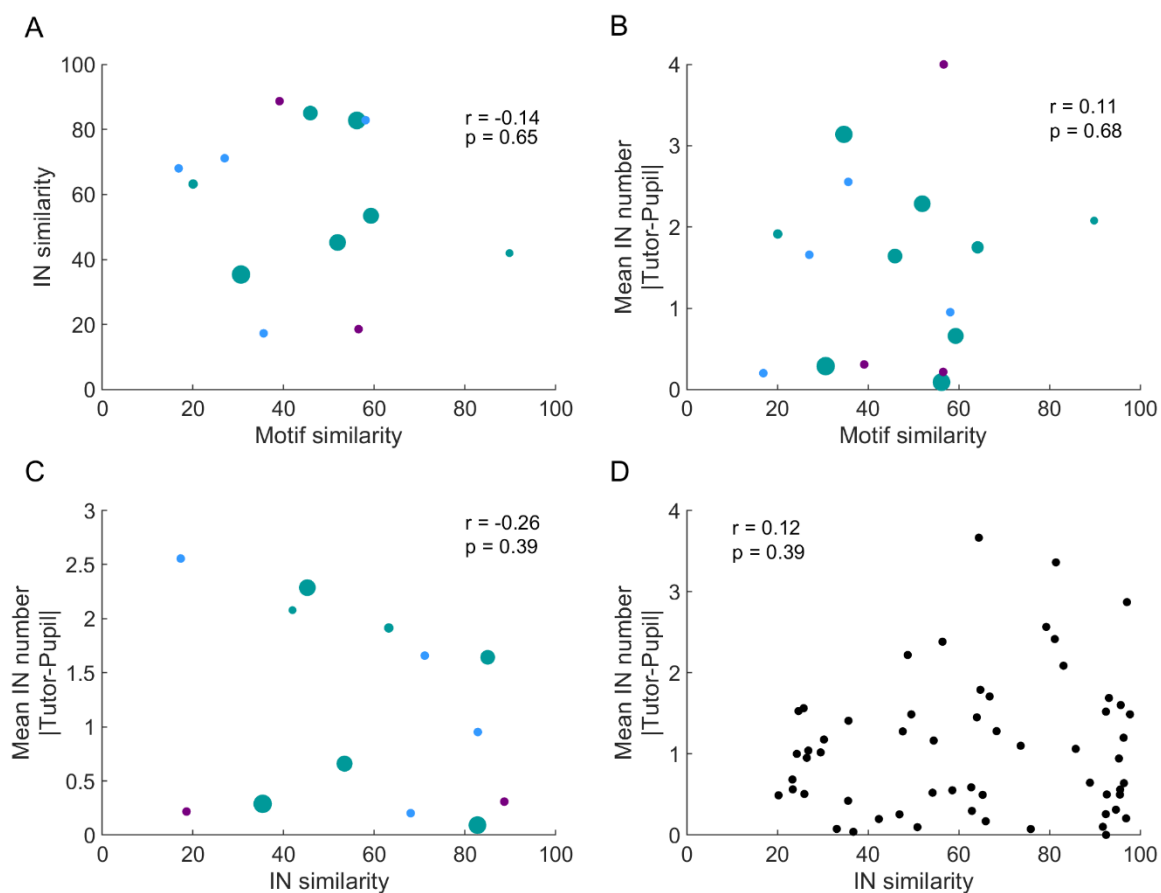


Figure 4.8 Learning of IN structure, but not number might be affected influenced by LMAN

Box plot of motif (A) and IN (B) similarity for different categories of birds namely, <20% lesion (n=6), 20-60% lesion (n=7), undetermined (UD) (n=3) and socially tutored birds (ST) (n=14). Number in bracket show number birds for motif similarity. Number of birds for IN similarity are, <20% lesion (n=6), 20-60% lesion (n=5), UD (n=2) and ST (n=10). Mean IN number of tutor vs pupil for birds with <20% lesion (C), 20-60% lesion (D), socially tutored birds (E). Each circle represents a birds. Pearson correlation coefficient was calculated. Size of circle reflects extent of lesion. Different color represent different groups of birds based on area of LMAN left. Red circles are birds for which amount of lesion could not be determined.

between tutor and pupil (Figure 4.8C). This also holds for normally reared birds (Figure 4.8D), suggesting that the mean IN number is learned independently of IN structure irrespective of the extent of LMAN lesion.

4.3.3 Why do birds with less damage to LMAN produce songs with lower similarity to the tutor's song?

Previous studies have shown that the effect of LMAN lesions also depends on the song produced by birds before LMAN lesions (Bottjer et al., 1984). LMAN lesion does not affect the similarity to the tutor for birds that have developed song similar to the tutor, prior to lesion. Thus, a bird with a higher LMAN lesion could have high song similarity to the tutor because it already had developed a similar song. To check if this could be why birds with 20-60% LMAN lesion show higher motif similarity than birds with <20% LMAN lesion. The box plot shows the prelesion similarity for all the groups of birds (Figure 4.9A). Irrespective of lesion size, the pre-lesion similarity lies in the same range. Birds with <20% lesion have a slightly lower median. Further, I calculated the correlation between song similarity before the lesion and the final crystallized song (Figure 4.9B). A linear trend can be observed irrespective of lesion size. However, the correlation was not significant. Thus, irrespective of lesion size, pre-lesion song

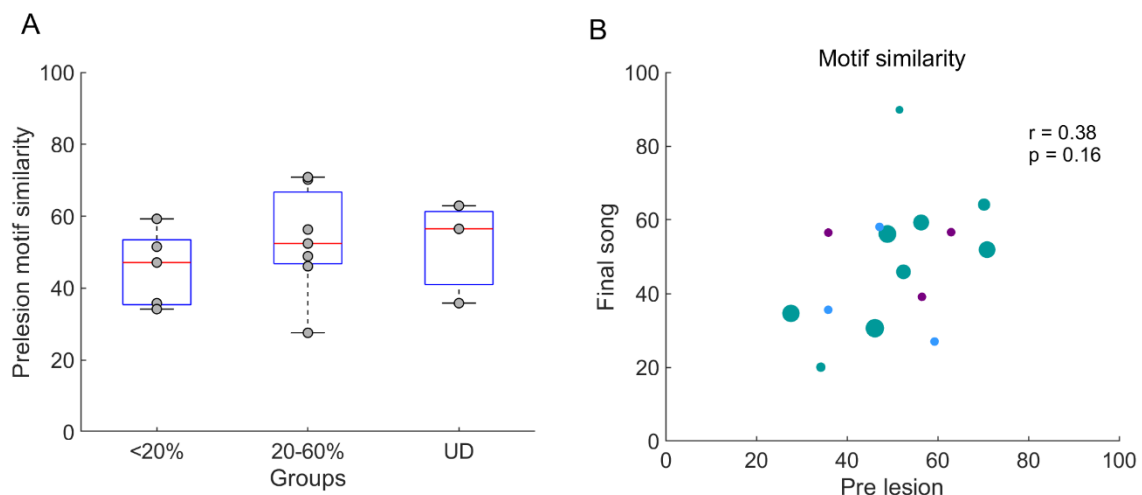


Figure 4.9 Motif similarity to the tutor's song before lesion might influence motif similarity after LMAN lesion.

A. Box plot for prelesion motif similarity between tutor and pupil for birds with <20% LMAN lesion (n=5), 20-60% lesion (n = 7) and undetermined (n=3). Each circle represents a bird. B. Plot of motif similarity to the tutor before lesion and the final song (n= 15). Each circle is a bird. Size of circle shows the extent of lesion. Red circles are birds for which amount of lesion could not be determined.

similarity has some effect on final similarity. This suggest that LMAN lesion might not have effect of accuracy of copying, if birds had developed song similar to the tutor before lesion.

4.3.4 Innate properties of IN, like temporal and acoustic progression, are not affected by LMAN lesion.

Next, I examined the effect of LMAN lesion on the innate properties of INs. I have previously shown that INs, like song, have both learned and innate properties (Chapter 2,; Kalra et al., 2021). Innate properties of INs include increased tempo (temporal progression), and the structure of INs becomes similar to the last IN (acoustic progression) as it progresses toward

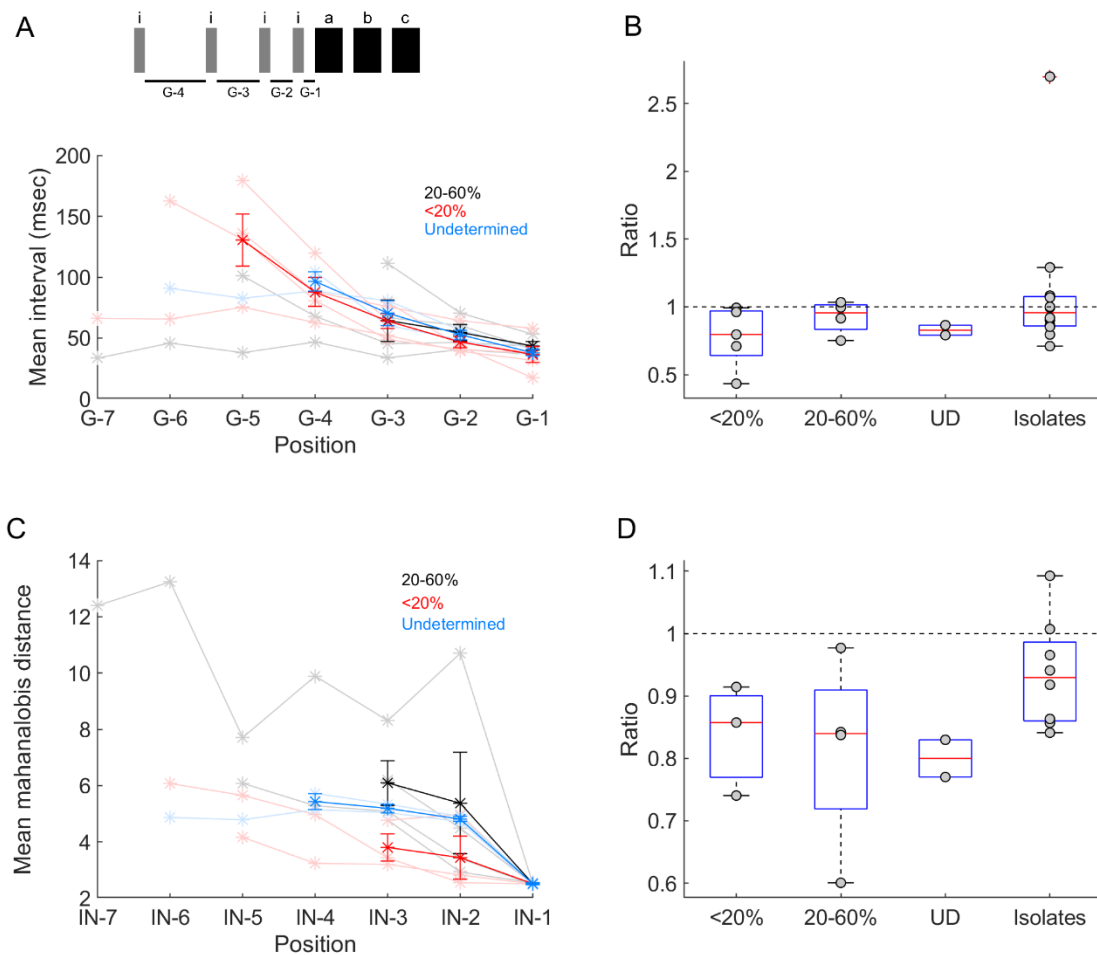


Figure 4.10 LMAN lesioned birds produce INs with temporal and acoustic progression

A) Top: Cartoon of song bout produced by zebra finch, grey represents INs and black motif syllables. Gap position labelled as G-1, G-2 are used in the below. Bottom: Interval between successive INs for bird with 20-60% lesion (black), (<20% lesion (red), (undetermined (blue), (n=2). B) Box plot of ratio of interval for all groups of birds. C) Mean Mahalanobis distance for birds with 20-60% lesion (black), (<20% lesion (red), (undetermined (blue), (n=2). D) Box plot of ratio for Mahalanobis distance for all groups of birds. For panel A and C, Lighter shades represent mean across bouts for individual birds and darker line is used for mean across birds. For B and D each circle represents a bird. Dotted line is used for marking $y=1$.

the song (Chapter 3). One would not expect them to be affected by LMAN lesion, given that LMAN is involved in learning. So, I compared temporal and acoustic progression in LMAN lesioned birds with that of isolates (birds reared without exposure to song and INs). The interval between the successive INs reduces for birds in all categories (Figure 4.10A). In order to quantify temporal progression, the mean ratio between successive intervals between INs was calculated (Chapter 3, See Methods, Figure 3.1A). A value less than 1 would mean that intervals between successive INs are reduced. Like untutored birds, this ratio was less than 1 for LMAN lesion birds (Figure 4.10B), suggesting that LMAN doesn't affect the temporal progression. Further, to check for acoustic progression, the Mahalanobis distance between all INs and the last IN for all groups of birds was calculated (see Methods Chapter 3). The Mahalanobis distance reduces towards the last IN for all groups of birds (Figure 4.10C). Similar to the temporal progression, the ratio for all groups of birds is less than 1 (Figure 4.10D), showing that LMAN lesioned birds also produce INs with acoustic progression. Thus, similar to untutored birds, birds with LMAN lesion show both temporal and acoustic progression. Overall, the absence of the effect of LMAN on temporal and acoustic progression suggests that LMAN does not mediate these properties.

4.4 Discussion

Introductory gestures precede many displays. However, we know less about the origin of introductory gestures preceding learned displays. Zebra finch, an Australian songbird, learns songs from its father or tutor (Immelmann Klaus, 1969; Marler, 1990), and the song initiates with INs (introductory gestures) (Price, 1979; Sossinka & Böhner, 1980; Zann, 1996). Further, INs, like a song, has both learned and innate components (Chapter 2; Chapter 3; Kalra et al.). Here, I have looked at the role of LMAN in learning the structure and mean number of INs before the start of the song. LMAN is involved in learning the structure and sequence of song (Bottjer et al., 1984; Scharff & Nottebohm, 1991), and I hypothesized that it might also be involved in learning the properties of INs. Birds with 20-60% LMAN lesion had lower IN structural similarity to the tutor compared to birds with <20% LMAN lesion. While the copying of mean IN number didn't seem to be affected by the lesion. Furthermore, birds with <20% LMAN lesion produced song with lower similarity to the tutor than birds with 20-60% LMAN lesion. While IN structural similarity was lower for birds with < 20% LMAN lesion. Furthermore, the lesion didn't seem to affect learning of the mean IN number. No correlation was observed

between IN number/ structure similarity and motif similarity, irrespective of the extent of the LMAN lesion. Innate properties of INs, like temporal and acoustic progression, were not affected by LMAN lesion. Overall, learning of IN structure but not number may be influenced by LMAN. However, due to less number of birds with LMAN lesions and incomplete lesions, these are not statistically significant.

4.4.1 Caveats

I would like to state the following caveats related to this study.

1. Here, I have quantified the amount of LMAN lesion using bright field microscopy of wet sections mounted directly on slides without staining. Use of different staining techniques like IHC or tracer injection in RA (LMAN projects to RA) might help to accurately quantify the LMAN area. I had used IHC and cresyl violet, but could not reliably mark LMAN in my experiments. In addition to this, I had only seven birds with unilateral LMAN lesions. In order to check if learning of IN number involved LMAN, one would require at least 14 birds (similar to the socially tutored birds, Chapter 2, Figure 2.5) with complete LMAN lesions. However, I had only 7 birds with 20-60% LMAN lesion.
2. I had lesioned LMAN around 46 days post-hatch (range 47-54 days post-hatch). Some birds already had developed songs similar to the tutor. Thus, it is difficult to discern the role of LMAN lesions in such cases on learning of IN number and structure. Previous study had suggested that around 10 days exposure to tutor's song is required for accurate copying of the song by juvenile zebra finch (Roper & Zann, 2006). Hence, I had performed lesion around 46 days post-hatch allowing 10 days exposure to the tutor's song (35 to 46 days post-hatch). One can lesion before day 46 to be sure that song is not stereotype prior to lesion. In order to control for number of days required for learning of INs, one can have different groups of birds, that are exposed to the tutor for different number of days.

4.4.2 A potential reason for birds with larger LMAN lesioned birds to have higher song similarity to the tutor.

As mentioned above, my results suggest that the extent of lesion of LMAN influences the accuracy of copying songs by juvenile male zebra finch from the tutor. In my study, birds with < 20% LMAN lesion had lower song similarity to tutor when compared to birds with 20-60%

LMAN lesion. Why do birds with smaller lesions of LMAN have lower similarity to the tutor than birds with larger lesion? Here, I put forward 3 possible hypotheses to explain this unexpected result. Firstly, it could result from a lesion in another brain region like mMAN (Bottjer, 2001) and/or AreaX (Scharff & Nottebohm, 1991; Sohrabji et al., 1990), which have been shown to affect song learning. These brain regions could not be quantified in wet sections and require more specific staining like immunohistochemistry using CGRP antibody, which marks these nuclei (Bottjer et al., 1997). Second, the LMAN to RA track could have been affected, which again will not show up in my sections used for quantifying the extent of the lesion. Injecting tracer in RA, can provide information regarding tracks as RA receives projections from LMAN (Bottjer et al., 1989). Third, most birds had unilateral lesions. Studies showing the role of LMAN on learning have performed bilateral LMAN lesions; on the contrary, most of the birds in this study had unilateral LMAN lesions. Thus, it could be another reason for the discrepancy in results.

4.4.3 Independent learning of IN number and structure.

Birds with 20-60% LMAN lesion show low IN similarity compared to other groups, while there is a positive trend for mean IN number between the tutor and pupil irrespective of the lesion size. Thus, it is possible that following the LMAN lesion, zebra finches copy the mean IN number from the tutor but not the IN structure. This suggests that learning of IN number and structure are independent. This is true for normally reared and socially tutored birds (Chapter 2). An earlier study has shown basal ganglia to be involved in learning time but not the acoustic feature of a syllable-like pitch (Ali et al., 2013). Similarly, LMAN may influence learning of IN structure but not the number. Another reason for this differential effect of LMAN on the number and structure of IN could be due to less tutor exposure before the LMAN lesion. Since birds had been exposed to the tutor from post-hatch day 35 to post-hatch day 46, this duration might not be sufficient to learn IN structure but enough to learn IN number. This could be tested by performing LMAN lesions on different days during the song-learning phase, which will be required to address these issues.

4.4.4 Development of IN number and structure during song learning.

Song learning involves the formation of a template for individual syllable structure and sequence information. Similarly, learning INs requires information about the structure and

number of the juvenile zebra finch. My results suggest that both structure and number of INs are learned independently. If so, birds should be able to change numbers and structure independently. A previous study showed that when given a new song to learn from, juvenile zebra finch changes the syllable structure independently of sequence (Lipkind et al., 2013). Is this true for INs as well? This can be tested by using the sequential tutoring method. Juveniles can be initially tutored and trained on low IN number songs, and then it can be trained on high IN number songs. If juveniles can change number without affecting the structure or vice versa, it would suggest that birds can change the number and structure independently. The ability to modify the structure of INs without affecting numbers will suggest that they can be independently controlled, similar to the song sequence. Further, one can characterize changes in IN number and structure during the song-learning phase. This can be compared with the learning of motif syllables. Preliminary data suggests that juvenile zebra finch changes both the number and structure of INs during the song-learning phase (Appendix Figure A5).

4.4.5 Innate properties of INs suggest species-specific characteristics/required for progression to the song.

Properties of INs, like temporal and acoustic progression, present in birds reared without exposure to INs are unaffected by LMAN lesions. Next, one could ask how the bird keeps track of this progression. One candidate for this could be Uva a mid brain nucleus, which shows neural activity at the onsets of introductory notes and motif syllables (Danish et al., 2017; Moll et al., 2023; Williams & Vicario, 1993). Following disruption of Uva activity during singing, birds stop singing only at the end of the syllable, even if disruption occurs in the middle of syllable. Thus, it could be one of the brain regions that can be involved in maintaining the temporal progression of INs. INs produced by birds with unilateral Uva lesion lack temporal pattern and does not progress to a song (Coleman & Vu, 2005; Danish et al., 2017). Disruption of Uva activity during the interval between INs would shed some light on its function in temporal progression.

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Chapter 5

Understanding the function of introductory notes

This work was carried out in collaboration with Chitvan Chandolia and Mahima Gautam.

5.1 Introduction

Animals have developed different ways of communicating. Many of these displays begin with introductory gestures. Some examples of introductory gestures include introductory tail flicks/push-ups produced by some lizards before a head-bobbing display (Fleishman, 1992; Peters & Evans, 2003), introductory notes produced by some songbirds before their song (Price, 1979; Richards, 1981; Soma & Iwama, 2017; Sossinka & Böhner, 1980; Zann, 1996), and introductory vocalization produced by some frogs (Backwell, 1988) and toadfish (Thorson & Fine, 2002) before their advertisement/aggressive call. Different functions have been hypothesized for these introductory gestures in different animals. Some species of frogs initiate their aggressive/advertisement call with an introductory note (Backwell, 1988). Frog species *Hyla microcephala* (Schwartz & Wells, 1985) and *Hyla ebracatta* (Schwartz & Wells, 1985) produce an introductory note with a longer duration as the distance between males of the species decreases. While for some lizards, introductory tail flicks/push-ups have been suggested to function as an alerting component (Ord & Stamps, 2008). To classify something as an alerting component, it should function to gain conspecific attention, and animals should be able to modify it based on different conditions. For instance, jacksnipe lizards (*Amphibolurus muricatus*) increase the duration of introductory tail flicks in windy conditions (Peters et al., 2007). Anolis lizards increase the amplitude of introductory push-ups when the receiver is further away. Furthermore, in anolis lizards, under low light conditions, the orientation time of lizards towards the robotic lizard is lesser if the species-specific head bobbing is preceded by an introductory gesture (Ord & Stamps, 2008). In songbirds, different functions have been speculated for different songbird species. Similar to lizards, in rufous-sided towhees (a songbird species), it is thought to function as an alerting component before the main message. The response of towhees to the playback of degraded species-specific (song was degraded by the addition of reverberation) is higher if preceded by the introductory notes (Richards, 1981). In white crown sparrows (another songbird species), it has been suggested to function in species recognition. Juvenile white crown sparrows learn heterospecific songs if preceded by a conspecific introductory whistle, suggesting its function in species recognition (Soha & Marler, 2000). Thus, there are different hypothesized functions for introductory gestures. Even though they are all introductory gestures before a display, they could have very different functions depending on the animal/species. Therefore, it is necessary to study function in every case where it is encountered. I have used zebra finch, a well-studied Australian songbird (Zann, 1996), to test some functions of introductory notes.

Zebra finches initiate their song bouts with repetition of short-duration vocalizations, called introductory notes (INs), followed by a consistent sequence called a motif/song (Price, 1979; Sossinka & Böhner, 1980; Zann, 1996). Interestingly, birds reared without exposure to the song during the song-learning phase also initiate their song bouts with introductory notes (INs) (Kalra et al., 2021; Price, 1979; Chapter 2, 3). Zebra finch sings both in the presence (directed song) and absence of female (alone song). Compared to alone songs, the number of INs is higher in directed songs (Sossinka & Böhner, 1980). Further, isolate zebra finches (birds reared without exposure to the song during the song-learning phase) also increase the number of INs while singing to a female (James et al., 2018). This suggests that zebra finches have an innate tendency to initiate songs with INs and produce more INs when singing to a female. This increase in the number of INs in directed songs suggests that INs might have a communicative role, like gaining female attention, or females might prefer a song starting with a higher number of INs. Previous studies have shown that female zebra finches prefer directed over alone songs (Woolley & Doupe, 2008). However, the effect of the number of INs on female song preference is not known. In order to test if INs play a role in female preference, I played back the zebra finch song with and without INs to females and characterized the response. Female zebra finches responded equally to the playback of songs with and without INs. Besides this, male zebra finches also increase the number of INs when they sing in the presence of a juvenile (juvenile-directed song) (Chen et al., 2016). This increase in IN number might be a way of gaining juvenile's attention to the song and thus affecting accuracy to song copying. I looked at the effect of IN number on learning of song by juvenile zebra finch. The number of INs did not seem to affect song learning. Overall, INs do not seem to play a role in gaining female attention under normal conditions.

5.2 Materials and Methods

All procedures at IISER Pune were conducted after approval from the Institute Animal Ethics Committee (IAEC), IISER Pune, and were in accordance with guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), New Delhi, India.

5.2.1 Experimental birds

Lab-bred adult female zebra finches (>90 days post-hatch) were used for playback experiments. A total of 35 female adult zebra finches were used for this study. 12 of these birds were excluded as they didn't respond at all or responded only 1 day. 13 (9 unfamiliar, 4 familiar) birds were used for preference assay 1, and 10 birds were used for preference assay 2. 4 birds that were used for the familiar assay belonged to the same nest (Father's song was used to test the preference).

5.2.2 Playback and experimental design

Before initiating the playback experiment, female zebra finches were isolated for 2 days in a sound attenuation chamber in separate cages (2 birds per box separated by cardboard partition). Playback was done in a cage (dimension: 38x27x29 cm), placed in a sound-attenuation enclosure with the speaker in front (Figure 5.1A). For acclimatization, birds were kept in the setup 15 minutes prior to starting stimulus playback. For initial set of 4 birds, 3 session/day separated by 90 seconds for 3 days with inter-trial for 6 mins were conducted. As these seem to be getting habituated, number of session were reduced to 1/day and inter-trial interval was

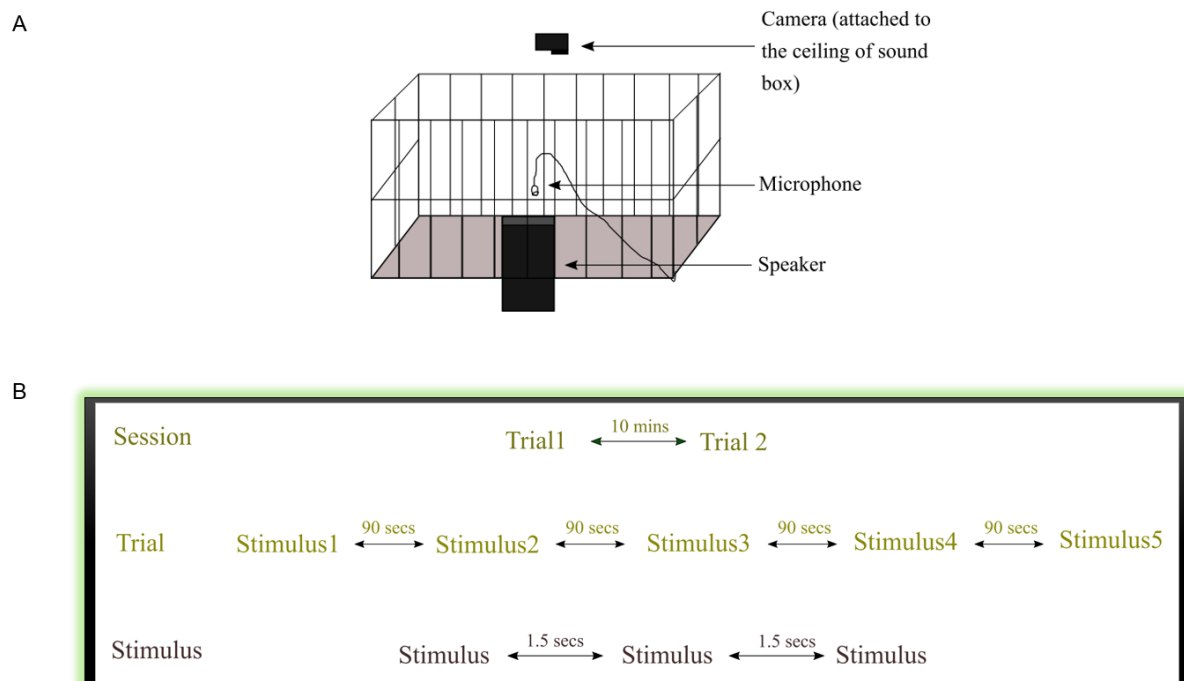


Figure 5.1 Experimental design

A) Cartoon representation of experimental setup. Speaker was placed in front of the cage and microphone attached to the same side of cage was used for recording sounds. GoPro attached on top of sound box was used for video recordings. B) Schematic of experimental design.

increased to 10 minutes for rest of the experiment. Each session consisted of two trials separated by 10 mins (Figure 5.1B). Stimuli were played in random order, separated by 90 seconds. Each category of stimuli had three repeats separated by 1.5 seconds of silence. Playback experiments were conducted for five days, interleaved by one day, to prevent habituation. A custom-written MATLAB script was used for the playback (CHANDOLIA, 2021). Different speakers were used for preference assay 1 and 2.

5.2.3 Different stimuli used for playback

Songs from two different adult male zebra finches were used to generate different stimuli for the playback experiment.

5.2.3.1 Preference assay 1 (Playback of a song with/without INs to familiar and unfamiliar female zebra finches)

The stimuli used in the first experiment to test the response of females to familiar and unfamiliar songs were generated from the song of same male. Different stimuli used for playback included normal zebra finch song (IN-Motif), only IN, only motif, reverse (Motif

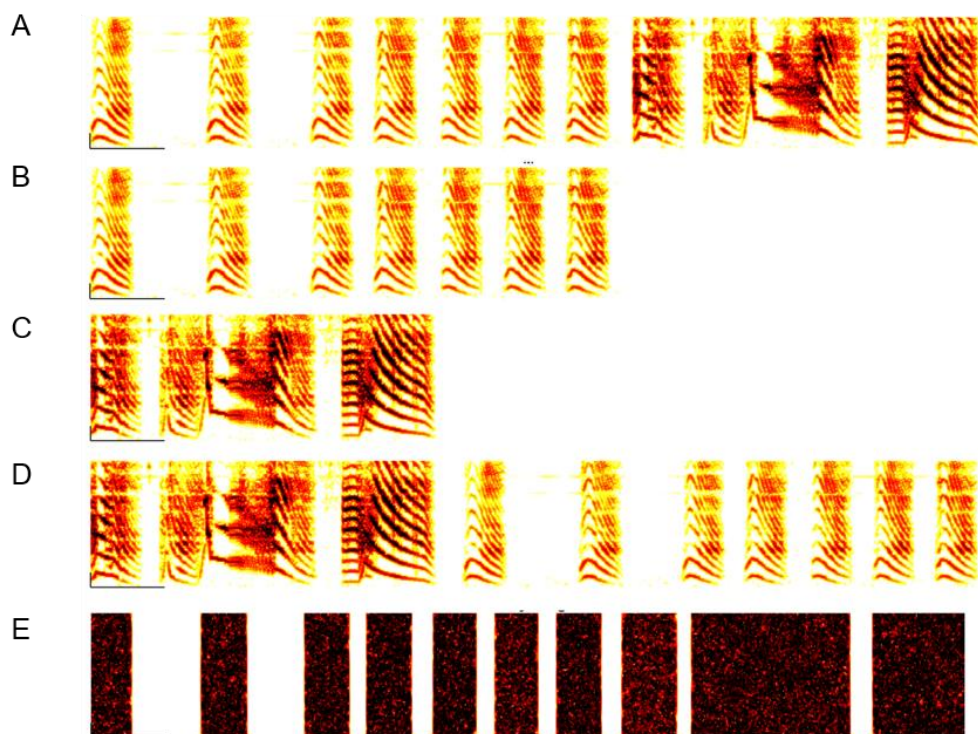


Figure 5.2 Spectrogram of stimuli used for preference assay 1

Spectrogram of normal zebra finch song (A) (IN-Motif), only IN (B), only motif (C), reverse (D) and noise (E). Scale bar: x-axis: 150 msecs, y-axis: 1000 Hz.

followed by INs), and noise used as a control (Figure 5.2). The noise was generated using MATLAB (MathWorks) function wgn (White Gaussian noise).

The noise stimulus was generated by replacing the acoustic features of the IN-Motif stimulus with white noise. The duration and order of syllables was kept the same. Reverse and noise were used as controls, reverse stimulus as a control for the duration of normal, while noise was used to as control for response to any sound.

5.2.3.2 Preference assay 2 (Increasing INs playback)

The song of another male zebra finch song and two repeats of motif was used to generate stimuli for this assay. To quantify the effect of increasing INs before motif, INs were artificially added before the motif. Rajan & Doupe (2013) showed that the interval between successive INs reduces towards the motif (temporal progression), and the amplitude of successive INs also increases (Rajan & Doupe, 2013). In order to maintain these properties, the interval between INs was determined by fitting an exponential curve to the mean interval. Data for the fitting curve were obtained from the songs of an unrelated set of birds (n=4) (Figure 5.3). Similarly, a linear fit was used for the amplitude of IN at different positions relative to motif onset.

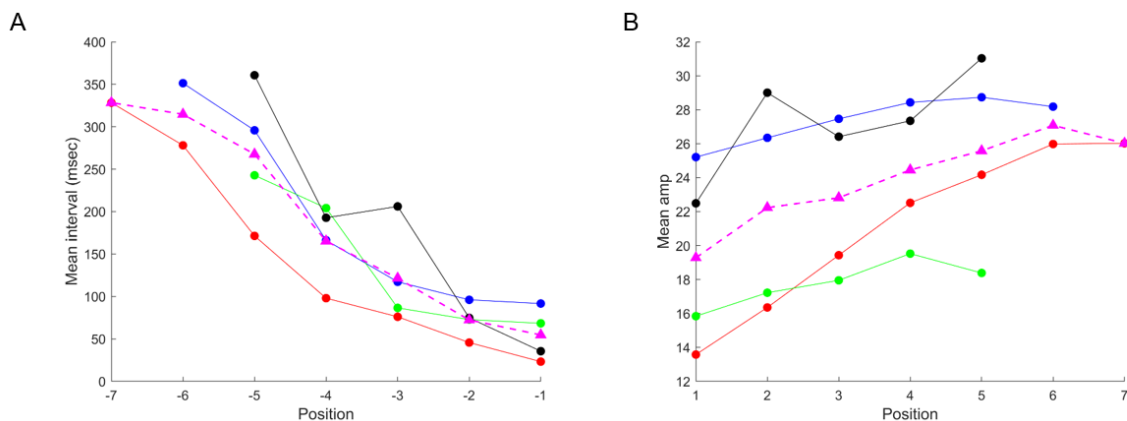


Figure 5.3 Mean interval and amplitude of INs for different birds

Plot of mean interval (A) and mean amplitude (B) for INs at different positions. Different colors represent different birds (n=4). Dotted magenta line represents mean across birds.

Different stimuli that were played back included (1) zero IN, (2) three IN, (3) six IN, (4) nine IN, (5) reverse (Motif followed by 5 INs), and (6) Noise (Figure 5.4). Noise stimuli were generated by adding random noise to 1000 Hz pure tone.

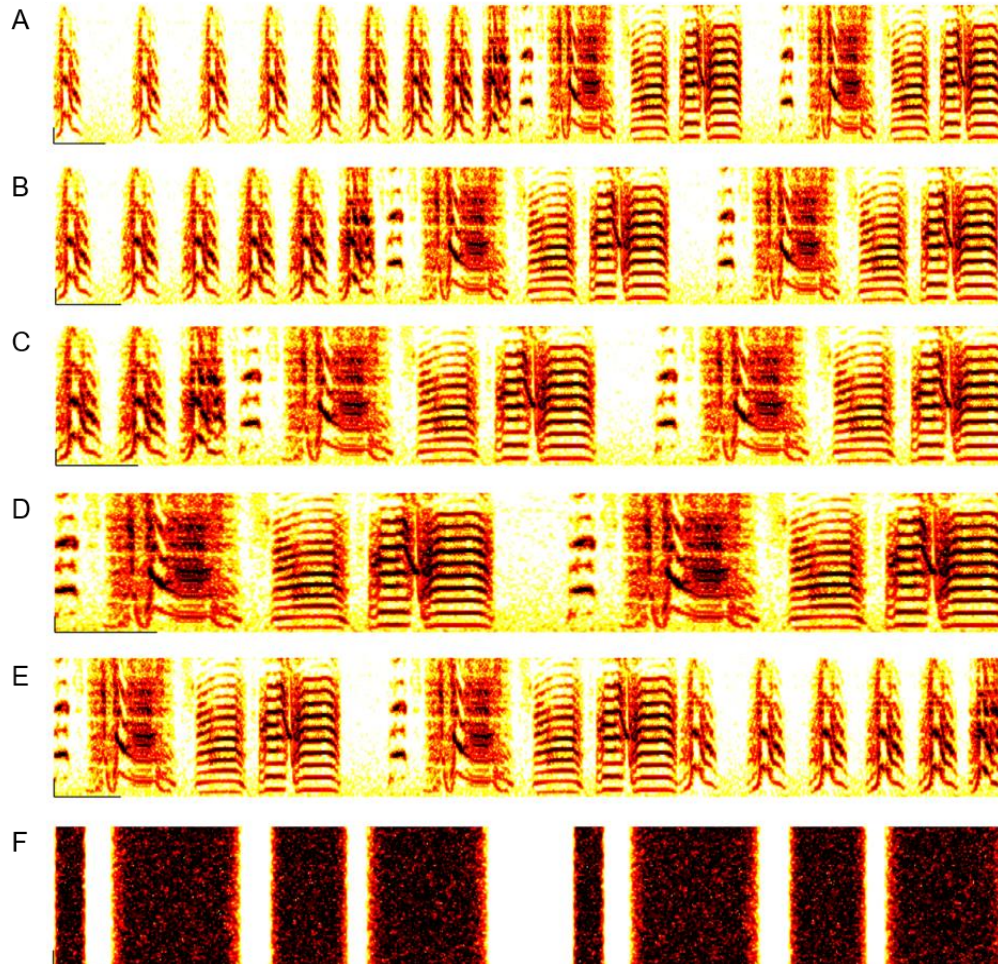


Figure 5.4 Spectrogram of stimuli used for preference assay 2

Spectrogram stimuli used for playback nine IN (A), six IN (B), three IN (C), zero IN (D), reverse (E) and noise (F). Scale bar: x-axis: 150 msec, y-axis: 1000 Hz.

2.4 Recordings

Songs were recorded in custom-made sound attenuation boxes (NewTech Engineering Systems, Bangalore, India) by placing an omnidirectional microphone (AKG 517) on top of the bird's cage towards the front of the cage. Signals from the microphone were digitized, and recorded on a computer at a sampling rate of 44100 Hz using a custom Python program. A continuous mode of acquisition was used for recording the sound. Video recording was done

only for preference assay one using GoPro (60 frames per second, 1080p resolution) directly on an SD card.

5.2.5 Analysis

All analysis was done using custom-written scripts in MATLAB (MathWorks). Panel function was used to generate figures for this chapter (Panel - File Exchange - MATLAB Central).

5.2.5.1 Segmentation and Annotation

Syllables were manually segmented and annotated using custom written MATLAB script.

5.2.5.2 Calculation of the mean number of calls produced by the female

In order to measure the female's response to the played-back stimuli, the number of calls produced during stimulus was considered. In some cases (preference assay 2; increasing IN playback), calls produced only during the motif were considered to understand the influence of INs on response to the motif. The mean number of calls for each stimulus was normalized by the duration of the stimulus.

5.2.5.3 Latency calculation

Latency was calculated as the interval between the onset of the stimuli and the onset of the first call produced by the female zebra finch.

5.2.5.4 Response probability

Response probability is defined as the probability of response to a particular stimulus. It is calculated using the following formula.

$$\text{Percentage response} = \frac{\text{Number of trial with atleast one female call}}{\text{Total number of trials}} \times 100$$

5.2.6 Statistics

All statistics was done using MATLAB. Repeated measures ANOVA was used for the comparison across groups. In cases where $p\text{-value} < 0.05$, Tukey-Kramer was used as post-hoc test to compare between groups. MATLAB function `ranova` was used to perform repeated measures ANOVA and `multcompare` for post-hoc. In order to perform repeated measure ANOVA for latency, trials where the birds did not respond, the latency was considered to be the duration of the longest stimulus (maximum latency). For correlation of song copying by juveniles and the mean IN number of the father, pearson correlation coefficient was calculated using MATLAB function `corrcoef`.

5.3 Results

5.3.1 *The presence of INs before the male zebra finch song does not influence females' preference*

In order to characterize the preference of female zebra finch for the conspecific songs with and without INs, I played back male zebra finch song stimuli through a speaker. As a measure of preference for each stimuli, I calculated the number of calls produced by the female zebra finch, which is a reliable measure of preference. Earlier studies have shown that the preference of females is maintained when tested using CSD as measure of preference or number of calls produced in response to the playback (Dunning et al., 2014; Nagle et al., 2002). Different stimuli used for playback were generated from the same male zebra finch song. These were 1) normal song (INs with motif), 2) only motif, 3) only INs, 4) reverse song (motif followed by INs) and 5) noise (see methods for details). Reverse and noise were used as a controls, reverse stimulus as a control for the duration of normal, while noise was used to as control for response to any sound. For all the different song stimuli, females responded maximally in the beginning (Figure 5.5A, Appendix figure; A6). In order to compare the mean number of calls produced by the female during the stimuli, I normalized the mean number of calls by the duration of stimuli. The mean number of calls produced by females was comparable across all song stimuli (Figure 5.5B). Noise had a significantly lower response ($p\text{-value}: 0.0079$, repeated measures ANOVA, post-hoc Tukey-Kramer). Further, I calculated the response latency, i.e., the interval between the start of stimuli and the onset of the first call produced by the female. Across the different stimuli except for noise, response latency was not significantly different (Figure 5.5C, $p\text{-value}: 0.0008$, repeated measures ANOVA, post-hoc Tukey-Kramer). I also quantified the

percentage of trials with a response. This was comparable for most song stimuli. Noise received the lowest response percentage (Figure 5.5D, p-value: <0.001, repeated measures ANOVA, post-hoc Tukey-Kramer). The response percentage for only motif stimuli was also significantly different from 'IN-motif' (p-value: 0.016, post-hoc Tukey-Kramer) and 'reverse' (p-value: 0.019, post-hoc Tukey-Kramer) stimuli. Overall, across different song stimuli there was no significant difference in female response. Equal responses to stimuli starting with and without INs suggest that females do not prefer songs starting with INs.

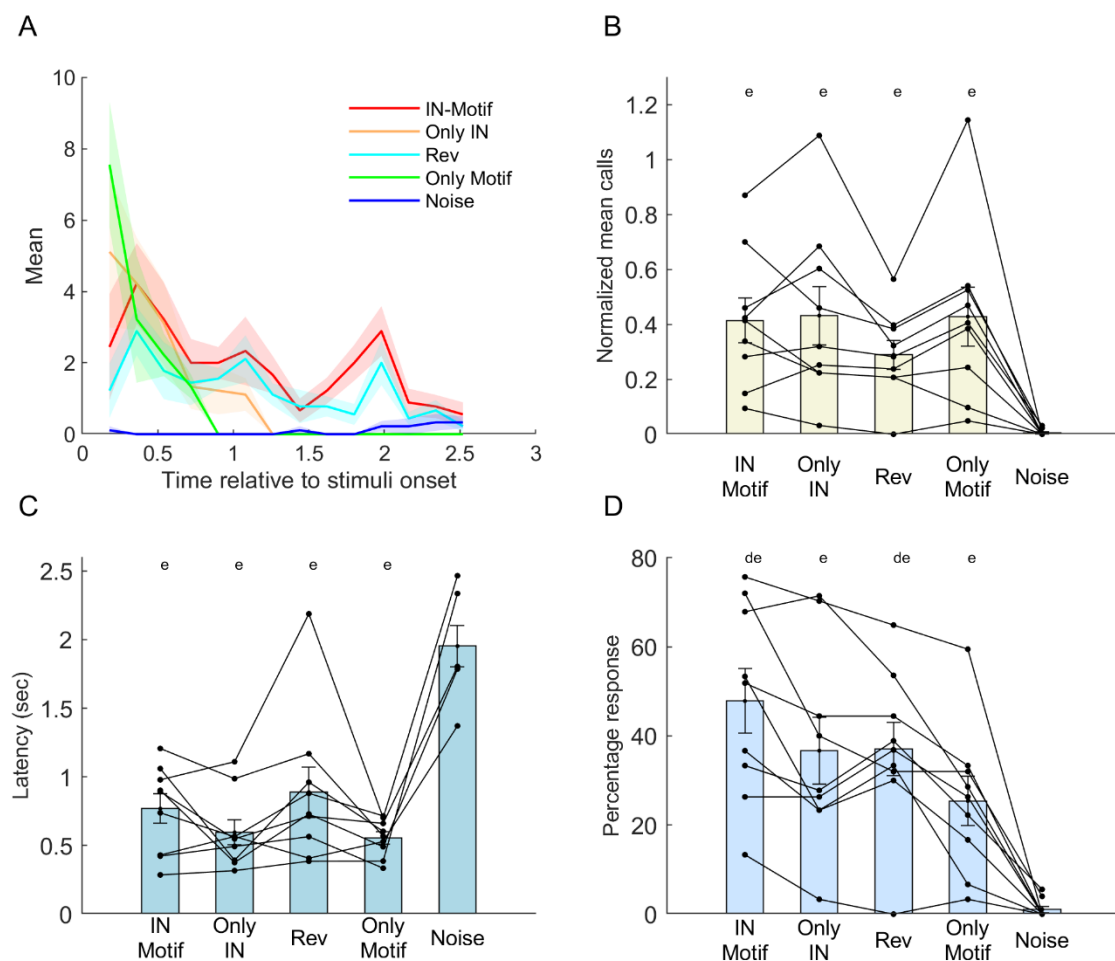


Figure 5.5 Female zebra finch does not show a preference for a conspecific song with INs

A. Mean number of calls across all females (n = 9), bin size 0.18 secs. Different colors represent different stimuli. Red: song with IN (IN-Motif), orange: Only introductory notes (Only IN), Cyan: Reverse song (Rev), Green: song without IN (Only Motif), and Blue: noise. The shaded region represents standard error. B, C, D. Bar graph for normalized mean number of calls (B), Latency (C), percentage response (D). Each bar represents different stimuli. Error bars are standard errors. Each point represents a bird. The line connects the same bird. Letters represent p<0.05, 'e' denotes all groups different from noise and 'd' marks all groups different from only motif.

5.3.2 The number of INs preceding the song doesn't influence female preference.

To further check if an extreme IN value (like 9) affects female preference, I played back the zebra finch song preceded by an artificially generated sequence of INs; IN numbers ranged from 0 to 9 in increments of 3. Similar to the above-mentioned results, female zebra finches responded equally to all stimuli. Their response was maximum at the beginning, irrespective of the number of INs (Figure 5.6A, Appendix figure, A7). The mean number of calls produced by female zebra finches during the motif was not significantly different across stimuli (Figure 5.6B, p-value: 0.7, repeated measures ANOVA). Similarly, response percentages for only the motif part of the stimuli (Figure 5.6D, p-value: 0.530, repeated measures ANOVA) and latency (Figure 5.6C, p-value: 0.565, repeated measures ANOVA) were similar across different song stimuli. The results further support the idea that INs before conspecific songs do not influence female preference.

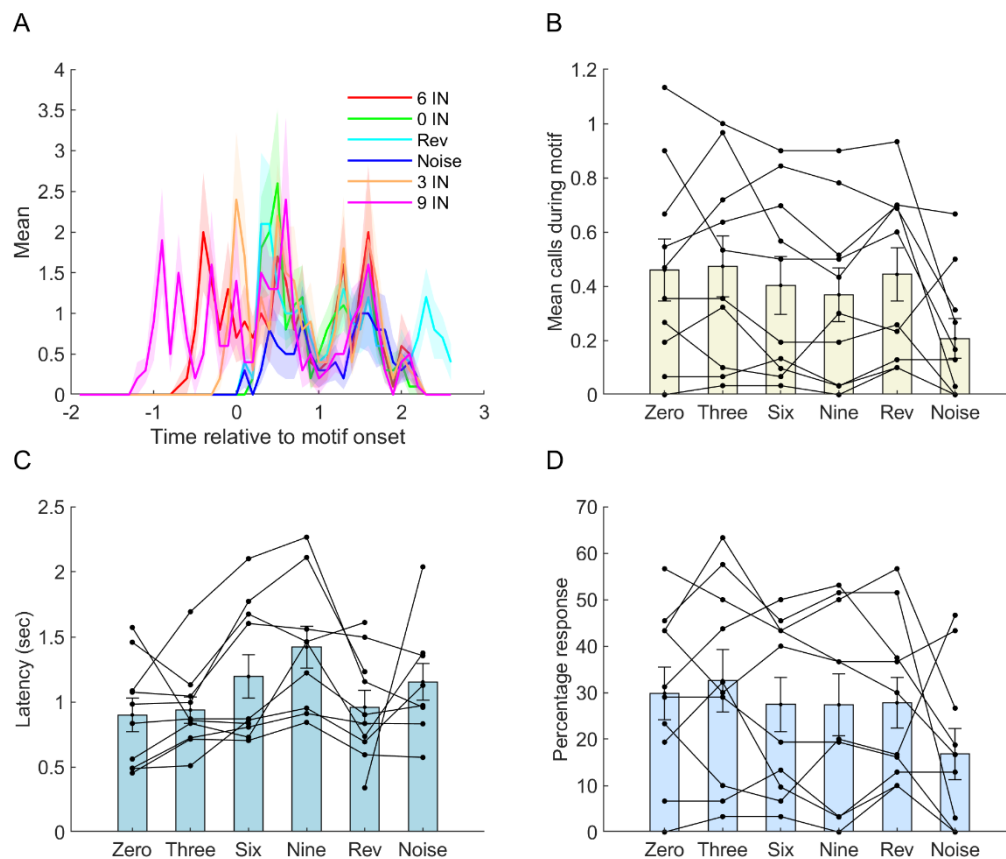


Figure 5.6. The number of INs before the song does not influence females' preference

A. Mean number of calls across all females ($n = 10$), bin size 0.1 secs. Different colors represent different stimuli. Red: song starting with 6 INs (6 IN), Green: song without IN (0 IN), Cyan: reverse (Rev), Blue: Noise, Orange: song starting with 3 INs (3 IN) and Magenta: song starting with 9 INs (9 IN). The shaded region represents standard error. B, C, D, Bar graph for mean number of calls produced during motif (B), Latency (C), percentage response (D). Each bar represents different stimuli. Error bars are standard errors. Each point represents a bird. The line connects the same bird.

5.3.3 Familiarity with the song does not influence the female zebra finch response.

An earlier study has shown that familiarity with song affects female preference. Female zebra finches prefer songs of father/ related males rather than completely unfamiliar songs (Riebel, 2000; Riebel & Smallegange, 2003). Thus, one reason for the absence of preference could be unfamiliarity with the played-back song. In order to test for the effect of familiarity on song preference, I played back songs of father with and without INs. Zebra finches responded equally to all stimuli. Females responded maximally in the beginning for all song stimuli played back (Figure 5.7A). Similar to the results mentioned earlier, the mean number of calls (Figure 5.7B, p-value: 0.088, repeated measures ANOVA) and percentage response (Figure 5.7D, p-value: 0.467, repeated measures ANOVA) were not significantly different across different song stimuli. Response latency was also not significantly different across stimuli (Figure 5.7C, p-value: 0.363, repeated measures ANOVA). Overall, they responded equally to songs with and

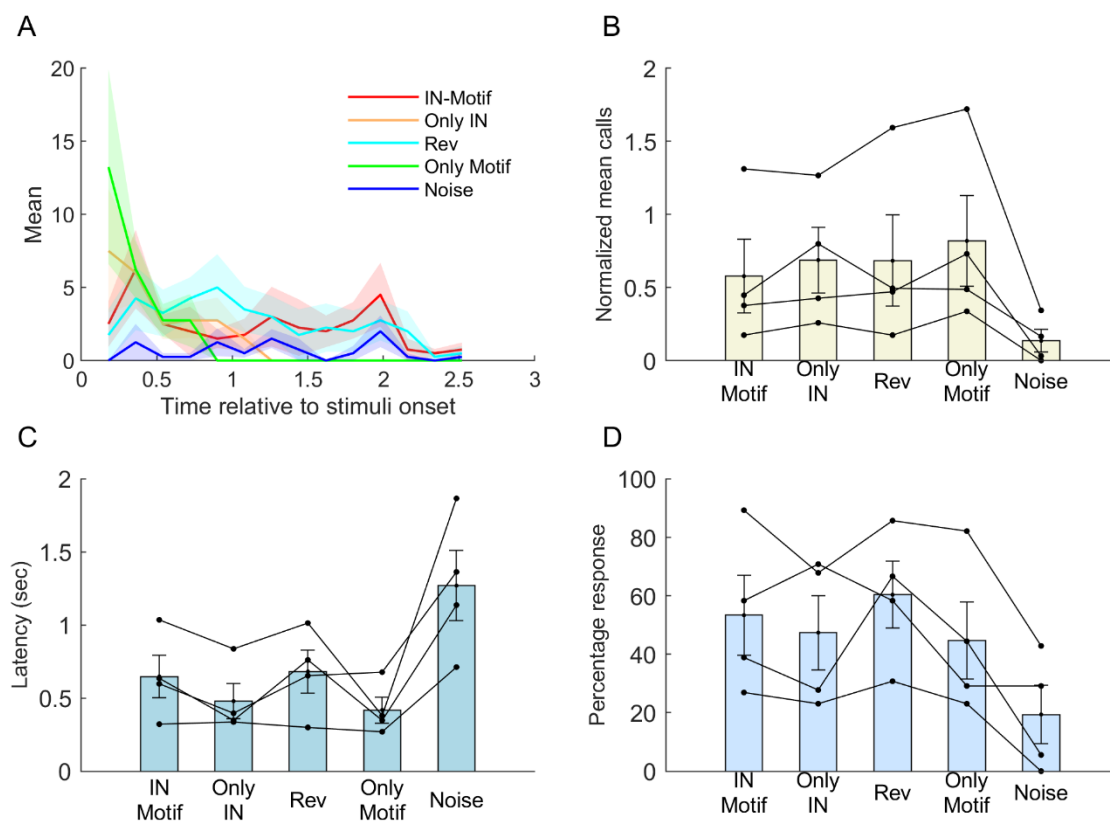


Figure 5.7. Familiarity to the song does not influence female's preference

A. Mean number of calls across all females ($n = 4$), bin size 0.18 secs. Different colors represent different stimuli. Red: song with IN (IN+Motif), orange: Only introductory notes (Only IN), Cyan: Reverse song (Rev), Green: song without IN (Only Motif), and Blue: noise. The shaded region represents standard error. B, C, D, Bar graph for normalized mean number of calls (B), Latency (C), percentage response (D). Each bar represents different stimuli. Error bars: standard errors. Each point represents a bird with line connecting same bird.

without INs, suggesting familiarity with the song did not affect the conclusions that INs do not influence female preference for a song.

5.3.4 *The presence of introductory notes does not influence the accuracy of motif copying.*

As mentioned before, adult male zebra finches increase the number of INs when they sing songs directed to a juvenile zebra finch. Increased IN number could make the juvenile pay more attention to the song. Therefore, I wanted to check if the IN number influences the accuracy of song copying. I found no correlation between the mean IN number of fathers and motif similarity between father and pupil (Figure 5.7, $n = 65$, $r = 0.08$, $p = 0.524$, Pearson correlation coefficient; see methods for details of motif similarity measurements). The absence of correlation suggests that INs don't affect accuracy of copying by the juvenile zebra finch.

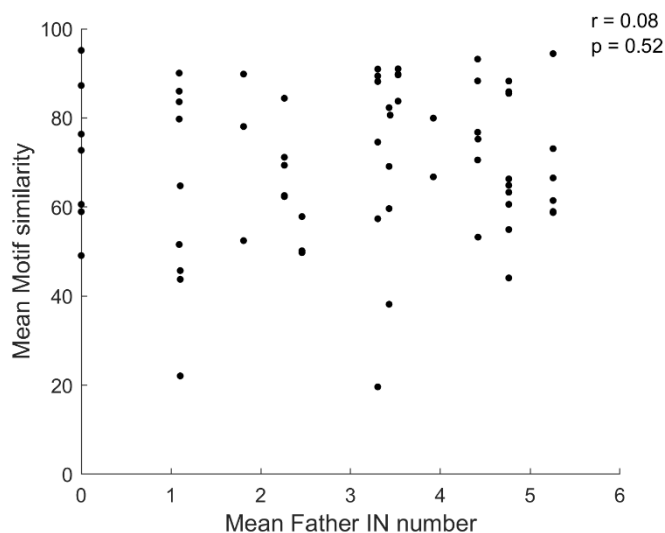


Figure5.8. Motif similarity between the father and pupil's song is not influenced by father mean IN number

X-axis represents the mean IN number of fathers, y-axis represents the mean motif similarity between the father and pupil's song. Each point represents a bird ($n = 65$).

5.4 Discussion

Introductory gestures have been suggested to perform different functions across different animals. I have used the zebra finch as a model system to examine the function of introductory gestures (INs). To test if a female zebra finch prefers songs starting with INs, zebra finch songs with and without INs and varying numbers of INs were played back to a female zebra finch through a speaker. Calls produced by the female in response to the playback were used as a measure of preference for particular stimuli. Songs starting with and without INs received comparable calls and the latency to calls was also comparable. Familiarity with the song did

not affect the female's response to different stimuli. Further, a previous study has shown that adult zebra finches produce song with more INs when singing to the juvenile (James et al., 2018). I have tested if number of INs produced by the male zebra finch affect accuracy of song copying. However, I did not find any correlation between the father's mean IN number and the accuracy of motif copying by the juvenile. Overall, my results suggests that females do not have a preference for songs starting with INs and presence of INs does not influence song copying by the juvenile.

Caveats associated with the measurement of preference

In order to measure the preference of zebra finch females for male songs with and without INs, I have used only calls produced by females in response to playback as a measure of preference. However, it is possible that other responses of females, like moving towards the speaker or performing a copulation solicitation display (CSD) along with calls produced, might give a better idea about female preference (Fujii et al., 2022; Zann, 1996). This will require analysis of video recordings during playback sessions. Many females didn't not respond in the preference assay (23/35 bird responded). One way of enhancing female response could be using a video of a male zebra finch rather than just a speaker. It is possible that physical presence of male motivates female to respond. Another way to test for preference could be perch hop/string pull methods (Barr et al., 2021; Braaten & Reynolds, 1999). Different perches equipped with IR led and receiver can be used (Braaten & Reynolds, 1999), such that a playback of stimulus is initiated once the female bird land on it. Each can be associated with the playback of different songs. Thus, the maximally elicited song by the female either by string pull/perch hop, would likely to be its preferred song.

The function of INs as altering components under noisy condition

One of the suggested functions of introductory gestures is as an altering component in rufous-sided towhees. Rufous-sided towhees' song consists of an introductory note followed by trills. To test the function of introductory notes as an alerting component, Douglas G. Richards (1981) played back a degraded song consisting of degraded INs and degraded trills (a degraded song was generated by adding reverberation and filtered using a 3 kHz bandpass) and degraded trill with normal IN. Playback of degraded songs in wild elicited more responses from other towhees compared to degraded trills, suggesting that introductory notes might function as an

alerting component (Richards, 1981). An altering function has also been shown for some species of Anolis lizards. Anolis lizard, also produce introductory push-ups before a non-vocal, head-bobbing display, and these introductory gestures have been suggested to function as an alerting component. Ord and Stamps (2008) used a robotic lizard to test the function of introductory push-ups in *Anolis* lizards before the head bobbing display. They looked at the time taken by other lizards to orient towards the robotic lizard, displaying the species' typical head bobbing display with and without introductory push-ups. They found that the orientation time was similar under normal light conditions for displays with and without introductory push-ups. However, during dim light, the orientation time was less for displays, starting with introductory push-ups. Thus, suggesting the introductory push-ups function as an alerting component (Ord & Stamps, 2008). In both Rufous-sided towhee and *Anolis* lizard, introductory notes have been suggested to function as altering components under noisy environments (acoustic noise for towhee and low light conditions for *Anolis* lizards). In the case of the zebra finch, I have played back zebra finch songs with and without INs under normal conditions and saw no specific preference by any female zebra finch. It is possible that similar to rufous-sided towhee and *Anolis* lizards, INs might function to gain female attention in noisy environments. To test for this, I played back degraded zebra finch songs starting with normal or degraded INs under different noise conditions. A degraded song was generated by randomly shuffling the frequencies present in the original song. The presence of normal INs enhanced the response to degraded songs from the female zebra finch (Appendix Figure, A8). However, only one of the birds in the set responded to the stimuli, and more experiments are required to make stronger conclusions. Thus, further experiments are necessary to determine if INs might function as an alerting component in a noisy environment to gain female attention toward the upcoming song.

Female zebra finch responds with short latency to playback of a song.

It has been shown in java finches that females perform CSD before hearing the entire song (Soma & Iwama, 2017). In my experiment female zebra finches also seem to have shorter response latency (~0.8 secs) to the song than noise. This also means initial vocalizations are sufficient for females to recognize and respond to the song. The initial vocalization could be few introductory notes or first few motif syllables. Thus, it is possible under normal conditions that females do not look at the number of INs but instead use initial vocalizations (either IN or song) to identify a song.

The function of INs in recognizing species-specific song.

Previous studies have shown that adult male zebra finches reared without exposure to the song also initiate the song with the repetition of INs (Kalra et al., 2021; Price, 1979; Chapter 2; Chapter 3). This suggests that zebra finches have an innate tendency to initiate song with INs. It is possible that since zebra finches initiate songs with INs, it provides information about the species song. Previous studies have shown that female have preference for the song (Braaten & Reynolds, 1999; Clayton & Pröve, 1989). I want to check if female use INs to identify species specific song. To test this, I played back bengalese finch songs starting with zebra finch INs and zebra finch song starting with bengalese finch INs. I used normal songs of zebra finch and bengalese finch with INs as control. Females responded equally to all stimuli (Appendix Figure, A9). This suggests that female zebra finches might not be using INs for recognition of species recognition. However, it is possible that zebra finches in our colony are accustomed to hearing bengalese finch songs as both species are housed together in our colony. This experiment would have to be repeated with other unfamiliar species song.

The function of INs in male-male competition

In frogs, INs have been suggested to function in male–male competition (Littlejohn & Harrison, 1985). It is possible that INs produced by male zebra finches also have similar functions. If it is true, one would expect, male zebra finches produce more INs in the presence of another male. It would be interesting to see if the number of males in the vicinity also influences the number of INs produced. Currently it is difficult to record vocalization for multiple individuals as a same without mixing to vocalization produced by two individuals in close proximity. One way to interference could be to use head implanted mic (Suri & Rajan, 2018) or wireless mics (D’Amelio et al., 2017).

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Chapter 6

Summary and discussion

6.1 Summary of results

Many animals start their communicative displays with introductory gestures. Introductory gestures precede both learned and innate displays. Different functions have been suggested for introductory gestures (Ord & Stamps, 2008; Rajan & Doupe, 2013; Richards, 1981; Soha & Marler, 2000). However, the origin and properties of these introductory gestures are not fully understood. I have used the zebra finch as a model system to address some of these questions. The zebra finch is a well-studied Australian desert songbird (Zann, 1996). In this species, only males sing as a part of courtship display. These birds learn the song from the tutor, in most cases its father, during the first 90 days after that, the song does not change (Immelmann Klaus, 1969; reviewed in Marler, 1990). The zebra finch song starts with introductory gestures/introductory notes (INs), short-duration repeating vocalizations, and a consistent sequence of vocalization called song/motif (Price, 1979; Sossinka & Böhner, 1980; Zann, 1996). The presence of INs before learned song makes the zebra finch a good model system to study the origin and function of INs in the context of learned vocalization.

In order to understand the origin of INs, initially, I have compared the mean IN number and structure of IN between father and pupil. Further, using different tutoring methods, namely playback tutoring and social tutoring, I have shown that similar to the song, mean IN number and structure are learned. Similar to previous studies, I have shown that birds reared without exposure to song/INs during the song-learning phase also start their song with INs (Price, 1979). Furthermore, I have compared the properties of INs produced by birds reared with and without exposure to INs. INs produced by these birds differed from innate vocalizations like calls and were structurally different among individuals. Similar to normally reared birds, birds reared in the absence of exposure to INs also repeat INs a variable number of times and have properties like acoustic progression (INs become more similar to the last IN as it progresses towards song) and temporal progression (interval between successive INs reduces as it progresses to song). However, features like entropy, entropy variance, and pitch goodness differed from INs produced by normally reared birds.

This suggests similar to the song, INs have learned and innate properties. Next, I asked if a brain region involved in song learning also influences learning of INs. LMAN (lateral portion of the magnocellular nucleus of the anterior nidopallium) is one of the brain regions involved in song learning (Bottjer et al., 1989; Scharff & Nottebohm, 1991). To test if LMAN is also involved in learning of INs, I performed bilateral electrolytic lesions of LMAN in juveniles birds during

the song learning phase and compared the INs produced by these birds with their tutors. The similarity in IN structure to the tutor was lower in birds with 20-60% LMAN lesion than in birds with less than 20% lesion. While the mean IN number was similar to tutor in both groups of birds. Irrespective of lesion size, birds produced INs with acoustic and temporal progression. However, due to the low number of birds with lesions, it is difficult to conclude the effect of LMAN lesions on the accuracy of IN copying. Overall, INs, similar to the song, have both learned and innate components. Brain region involved in song learning might be involved in learning IN structure but not number.

Next, I looked at the communicative function of INs in zebra finches. The male zebra finch produces more IN when it sings to the female zebra finch (Sossinka & Böhner, 1980). This suggest INs might have a role in gaining females attention or female zebra prefers song starting with higher IN number. To test if a female prefers a song with a higher IN number, I played back a zebra finch song with varying IN numbers and used the calls produced by the female in response to playback as a measure preference. Female zebra finches responded equally to song starting with and without INs. The latency of response was similar for all stimuli. Thus, female zebra finches do not prefer songs with INs under normal conditions. Adult male zebra finches also produce high IN when singing to a juvenile male zebra finch (Chen et al., 2016). Thus, I next checked if the number of INs produced by male influences the accuracy of song copying by the juvenile zebra finch. However, the IN number does not seem to influence the accuracy of copying by juvenile zebra finches. Overall, these results suggest that female zebra finches do not prefer song with INs and under normal conditions, INs might not function to gain female attention.

6.2 Neural mechanism underlying IN production

In this thesis, I have shown that INs produced by zebra finches, similar to song have both learned and innate components. Learned properties of INs include the number and structure of IN. Further, variable number of repeats at the beginning of the song, temporal and acoustic progression are innate properties. Given that INs have both learned and innate components like song, it suggests common brain regions might be involved in their production. In fact, damage to brain regions that affects learned features of the song also affects INs. One such region involved in the production of song sequences is HVC (proper name). Cooling HVC, which results in changes in the duration of song syllables, also shows changes in the duration of INs

(Long & Fee, 2008). However, this change in the duration of INs is lesser than song syllables. Rajan & Doupe (2013) have shown that specific neurons are active during INs. They have shown that some of the AreaX projecting HVC neurons fire only for the last IN, and for some, the firing rate was different relative to the preceding IN (Rajan & Doupe, 2013). Overall, INs related activity has been shown in brain regions involved in song production. Besides this, HVC receives projection from other brain regions, one of them being Uva (Uvaeformis), a thalamic nucleus, which also affects song sequence production (Nottebohm et al., 1982). Similar to HVC lesions, following bilateral Uva lesions, birds produce subsong. Previous studies have shown that Uva is active at onsets of both song syllables and introductory notes. Further, following unilateral Uva lesions, birds produce strings of INs that do not progress to the song (Coleman & Vu, 2005; Danish et al., 2017; Moll et al., 2023; Williams & Vicario, 1993). Disruption of Uva activity during song production makes birds stop singing at the offset of a syllable. While following similar disruption in HVC, birds stop singing after a fixed interval following stimulation, even if this falls within the middle of a syllable (Ashmore et al., 2005; Moll et al., 2023). This suggests Uva might control the onset of syllables. If Uva controls the initiation of INs, it might also have a role in maintaining the temporal progression of INs. The function of Uva in IN production can be tested by performing microlesions in Uva or disrupting activity in Uva during the intervals between INs. Furthermore, Uva receives inputs from a midbrain nucleus, DM (dorsomedial nucleus of the intercollicular complex), which initiates vocalizations (Fukushima & Aoki, 2002; Striedter & Vu, 1998; Vicario & Simpson, 1995). It is also possible that DM influences the activity of the Uva neurons and, hence, the production of INs. Both DM and Uva also receive inputs from the respiratory center, PAm (nucleus paraambigualis) (Ashmore et al., 2005); thus, some feedback loop might be affecting IN production. This feedback loop might be required for initial INs, thus resulting in longer intervals. Neural recording in DM in awake singing birds or performing DM partial or complete lesions might help to discern its role in IN production. Overall, the brain region responsible for producing learned vocalizations, like songs and long calls, might also be involved in the production of INs.

6.3 Learning of IN number and structure

Male zebra finches learn their song from the tutor (mostly father) during the initial 90 days (Immelmann Klaus, 1969; reviewed in Marler, 1990). Different phases of song learning include

the sensory acquisition phase, during which the juvenile forms the template of the tutor's song. The sensory-motor phase follows this; during this phase, the juvenile tries to produce vocalizations similar to the tutor song, then the final song by day 90 (reviewed in Marler, 1990). Brain region like LMAN influences the learning of song syllable by the juvenile zebra finch. Lesion of LMAN during the song-learning phase affects the accuracy of song copying by the juvenile zebra finch (Bottjer et al., 1984; Scharff & Nottebohm, 1991). In my thesis, I have shown that, similar to song syllables, LMAN might be involved in learning of IN structure but not number. Further, the IN number is learned independently of the IN structure or song syllable. However, due to the fewer birds with lesions, the role of LMAN in learning of INs is inconclusive. Another region involved in learning song syllables is NIf, an auditory-motor region (nucleus interfascialis of the nidopallium). Lesioning NIf before tutoring affects the accuracy of copying song syllables by juvenile zebra finch (Roberts et al., 2012; Zhao et al., 2019). The involvement of NIf in learning of IN number and structure is not known. Some of the NIf neurons in juveniles are active for specific syllables produced by the tutor (Mackevicius et al., 2020). It would be interesting to see if NIf has neurons specific for the tutor's IN number/structure. Apart from this, stimulation of HVC while the juvenile listens to the tutor's song also affects the accuracy of copying (Roberts et al., 2012). Thus, HVC might be another region coding for IN number/structure. It would be interesting to see if stimulation in the HVC of juvenile zebra finch while listening to INs affects its learning. Furthermore, the absence of correlation between the accuracy of song copying and accuracy of IN number/structure copying suggests that IN number is learned independently of the song. Do birds recognize INs differently from songs? Bird tutored with a live adult male zebra finch produce INs preceding song; they maintain the order of INs and song. As mentioned, zebra finches have a biological predisposition to produce INs before the song. However, when given an opportunity to learn from the tutor, birds can copy the number and structure of INs. How do they distinguish between INs and motif syllables: birds don't use motif syllables instead of INs. What properties of INs do birds use to identify INs, or are they just learned as a part of a sequence? These are some of the interesting questions for future studies to elucidate more about the organization of song bouts with INs followed by song.

6.4 Function of INs

As mentioned above, there are different speculated functions of introductory gestures in various animals. In the case of lizards, under dim light conditions, it has been suggested to function as an alerting component (Ord & Stamps, 2008). However, for frogs it is suggested to be involved in male–male competition (Littlejohn & Harrison, 1985). In comparison, for various songbird species, different functions have been suggested for INs; alerting function in the case of towhee (Richards, 1981), species recognition for juveniles to learn correct song in white crown sparrows (Soha & Marler, 2000), and motor preparation in zebra finches (Rajan & Doupe, 2013). In my thesis, I have looked at the communicative role of INs. I have shown that female zebra finches don't have a preference for song starting with INs. However, preliminary data suggests that if a degraded song (produced by randomly shuffling the frequencies in a syllable) starts with normal INs, it seems to enhance the response to the upcoming song. But the playback experiment needs a greater number of birds. It is possible that similar to towhee, it might function as an alerting component before the song. Comparing properties of INs across different species of birds might shed light on common functions. Similar properties among songbird species might suggest a conserved trait and a similar function. While different features are likely to reflect the presence of a species-specific trait. Further, comparing the properties of these INs relative to the song/main message can provide some information regarding what makes IN suitable for a particular function. For example, lizards (*Amphibolurus muricatus*) use introductory tail flicks as an alerting component. These tail flicks are higher in amplitude and less constrained by viewing angle, making it an appropriate introductory component (Peters & Evans, 2007; Peters & Ramos, 2022). Similarly, in towhee, INs are composed of a tonal component with no amplitude modulation, making it less susceptible to reverberation compared to the trills (main message) (Richards, 1981). Thus, differences in properties of INs relative to the main message and comparison across species might also shed some light on its potential function.

6.5 Future directions

I have shown that zebra finches learned IN number and structure from the father. Further, zebra finch repeats INs a variable number of times before a consistent song. This makes INs a good candidate to study the production and function of introductory gestures. Whether INs have a role in initiation of learned motor movements remain to be determined. There are many open

questions in the field, like how birds keep track of IN number while singing, and the possible reason for birds producing variable number of INs. Furthermore, how juvenile zebra finches keep track and form a template of INs produced by their father. Overall, not much is known about the mechanism of IN production and learning and my thesis highlights the importance of studying INs before zebra finch song.

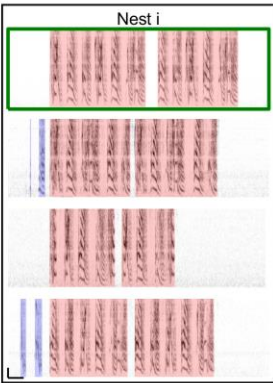
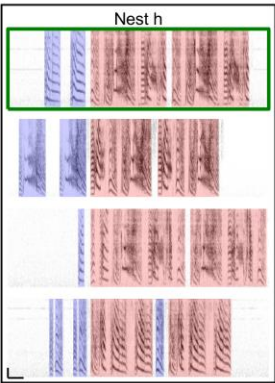
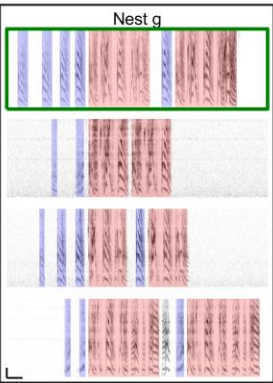
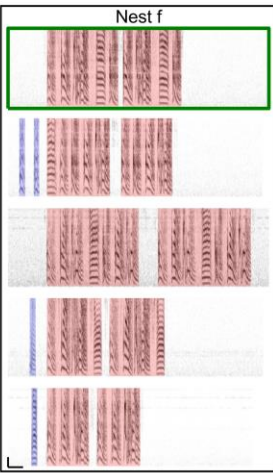
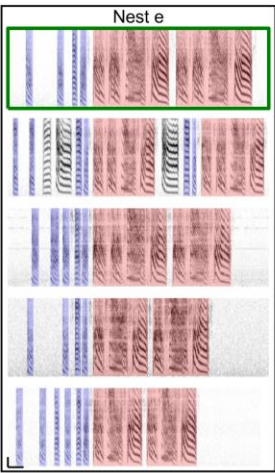
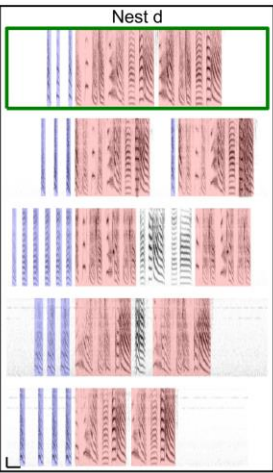
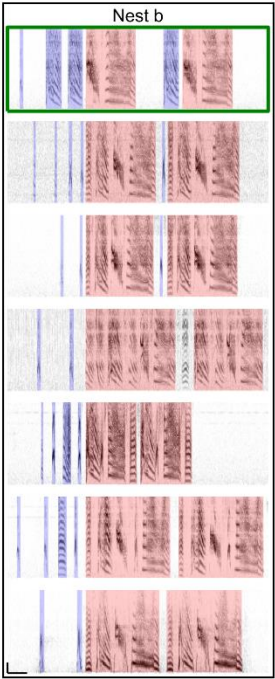
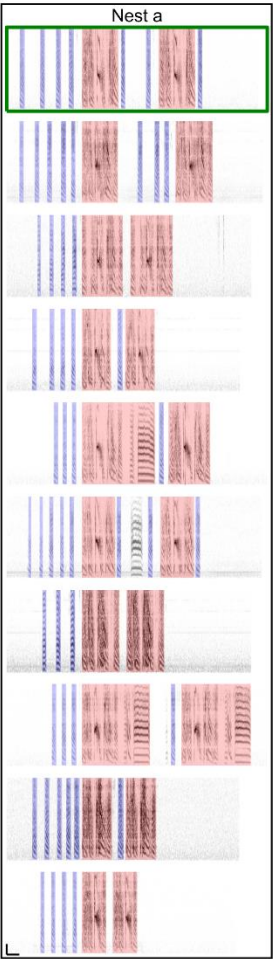
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Appendix



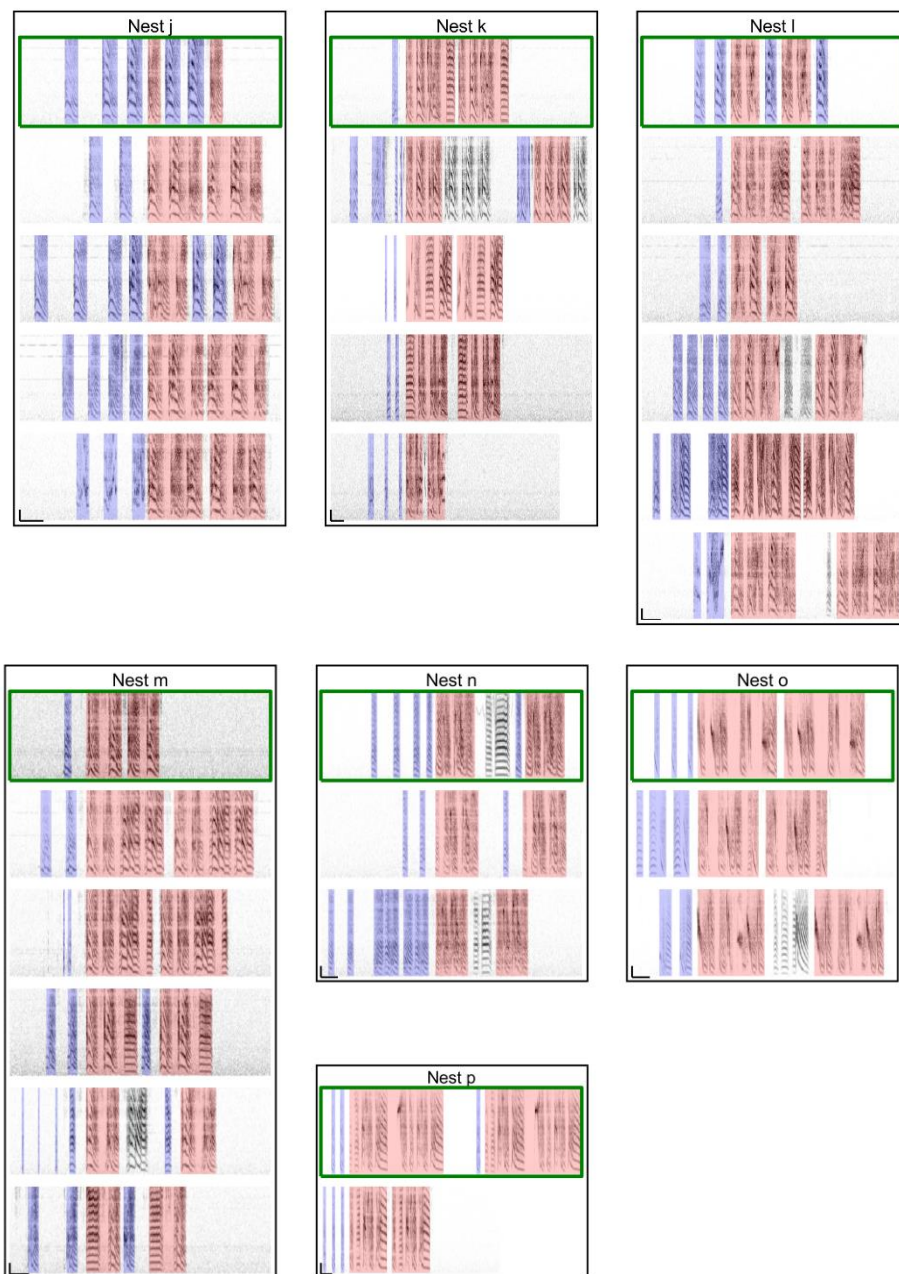


Figure A1 Spectrograms of fathers and their sons from all nests

Each vertical black box includes spectrograms of one song bout for fathers (top, enclosed within a green box) and their sons for one of the 16 nests used in our study (Nest a to Nest p). Blue shading highlights INs and red shading highlights motifs. Calls at the beginning of the bout or between motifs are not highlighted.

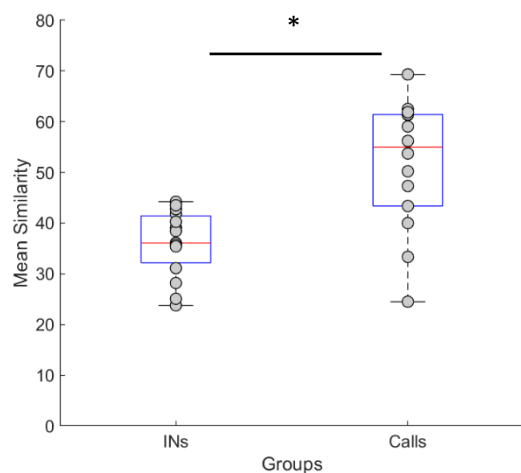


Figure A2 Box plot of mean similarity of INs and calls among isolates.

Box plot of mean IN and call similarity among isolates. Each point here represents a bird. * represent $p < 0.05$, Wilcoxon rank-sum.

Left hemisphere

Right hemisphere



Figure A3. Sagittal sections of brain section visualized using bright field microscope for bird with <20% LMAN lesion.

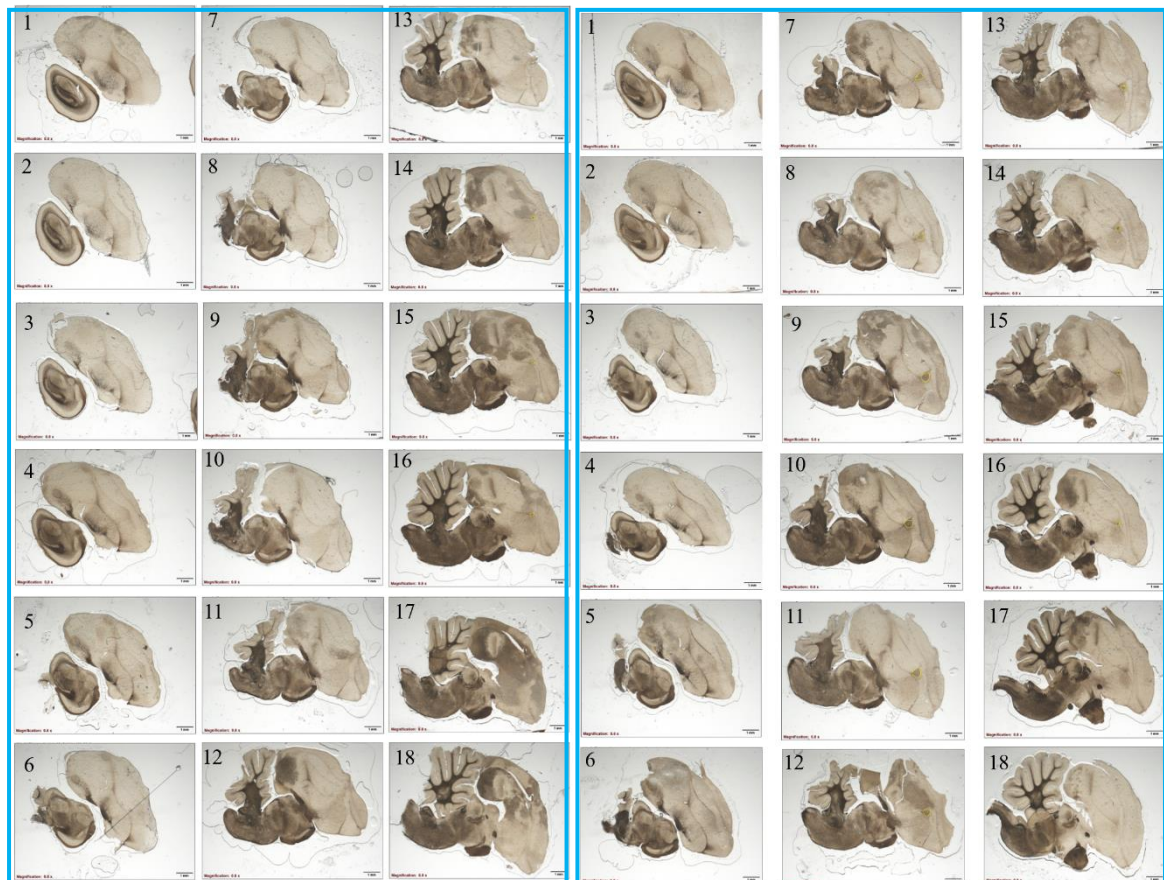


Figure A4. Sagittal sections of brain section visualized using bright field microscope for bird with 20-60% LMAN lesion.

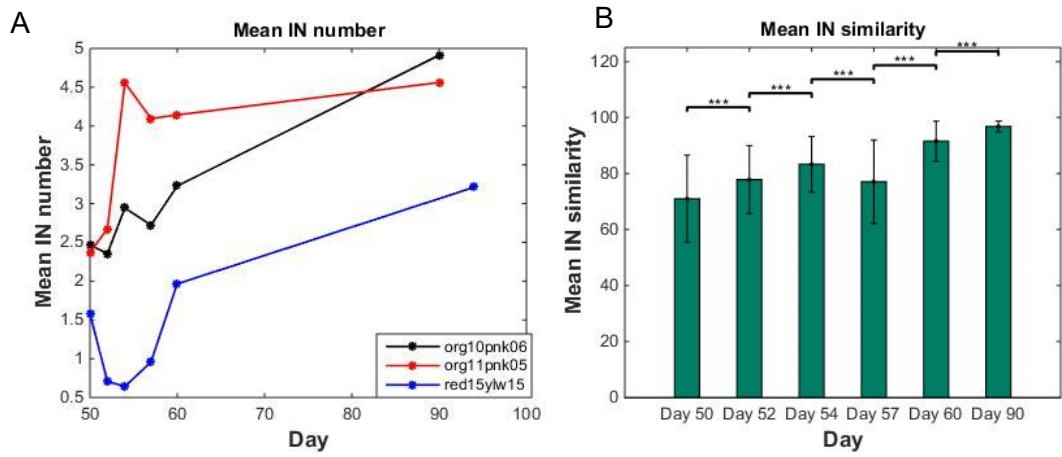


Figure A5. Changes in IN number and structure with age

A) Plot mean IN number with age. Different colors represent different birds (n=3). x axis represents post hatch day and y axis represent mean IN number. B) Bar graph of mean IN similarity with day 90 IN. Each bar here represents mean similarity for the mentioned day with phd90. Error bar – standard error.

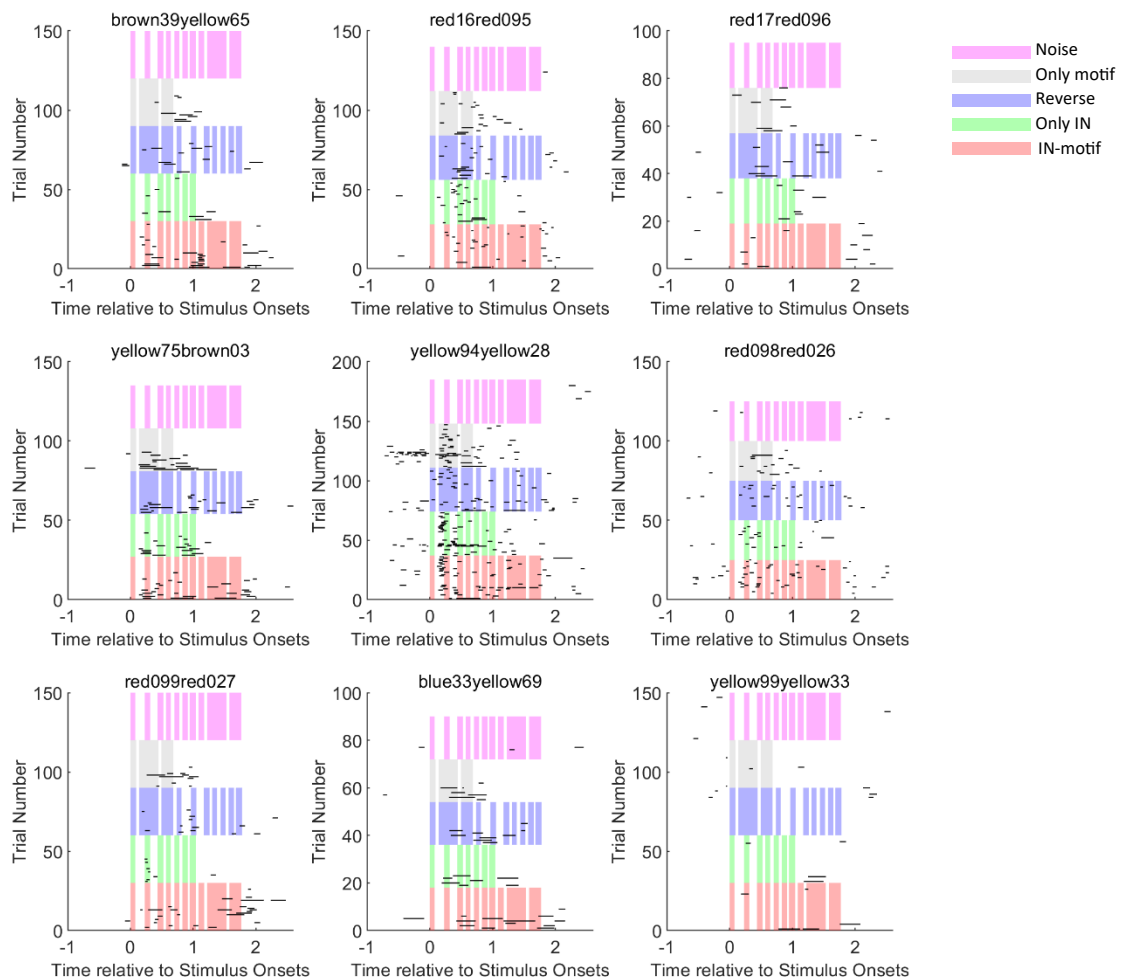


Figure A6. Raster plot for preference assay 1

Each plot here is for different birds. y axis represents the trial number and x axis represents time relative to the stimulus onset. 0 marks beginning of the stimulus. Black lines represent calls produced by the female. Shaded region shows the stimuli played back. Different colors represent different stimuli (Red: IN-Motif, Green: Only IN, Purple: Reverse, Grey: Only motif and Pink: Noise).

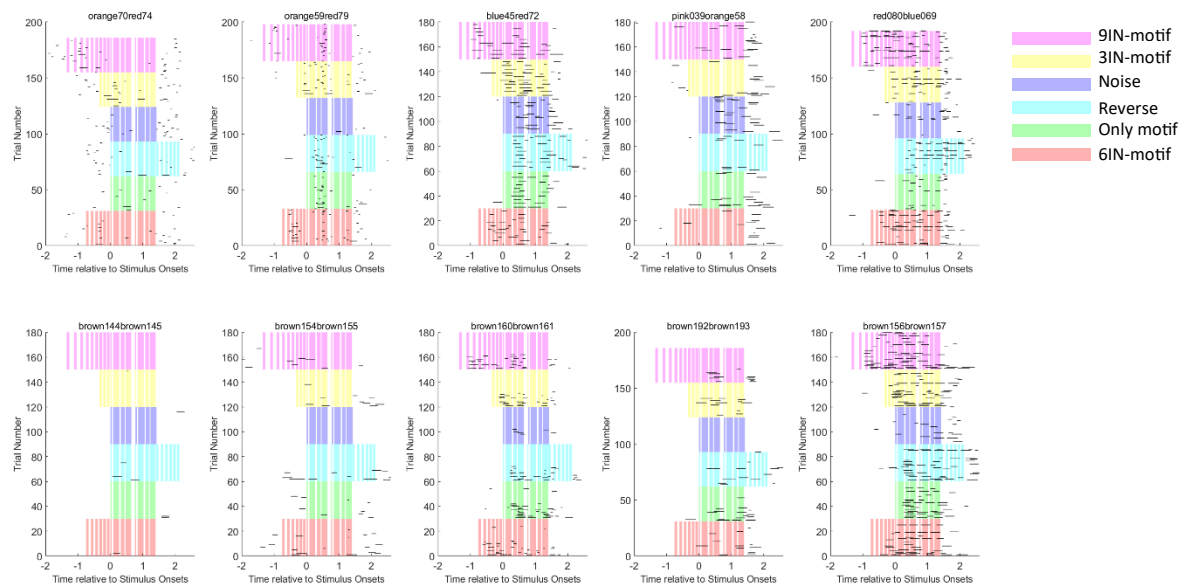
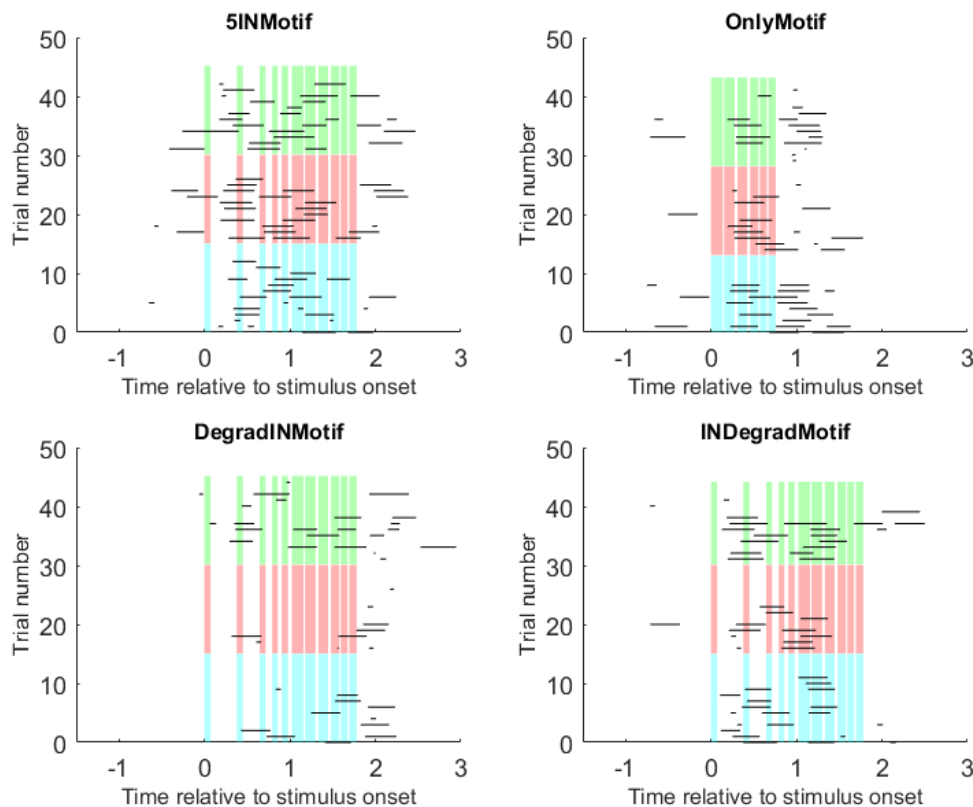


Figure A7. Raster plot for preference assay 2

Each plot here is for different birds. y axis represents the trial number and x axis represents time relative to the motif onset. 0 marks beginning of the motif. Black lines represent calls produced by the female. Shaded region shows the stimuli played back. Different colors represent different stimuli (Red: 6IN-Motif, Green: Only Motif, Cyan: Reverse, Purple: Noise, Yellow: 3IN-Motif and Pink: 9IN-Motif).



FigureA8. The presence INs before the degraded motif enhances the female's response to the zebra finch motif

A,B,C,D, Raster plots for calls produced by a female zebra finch in response to playback of zebra finch song consisting of normal INs and motifs (A), only zebra finch motif (B), Degraded zebra finch INs and motif C, Degraded zebra finch motif starting with normal INs. x-axis is time relative to stimulus onset, the y-axis represents trial number. Each black line is calls produced by the female zebra finch. Patches represent the stimulus played back. Different color patches represent different level of colony noise. Cyan: High noise, red: Low noise, green: silence. N =1

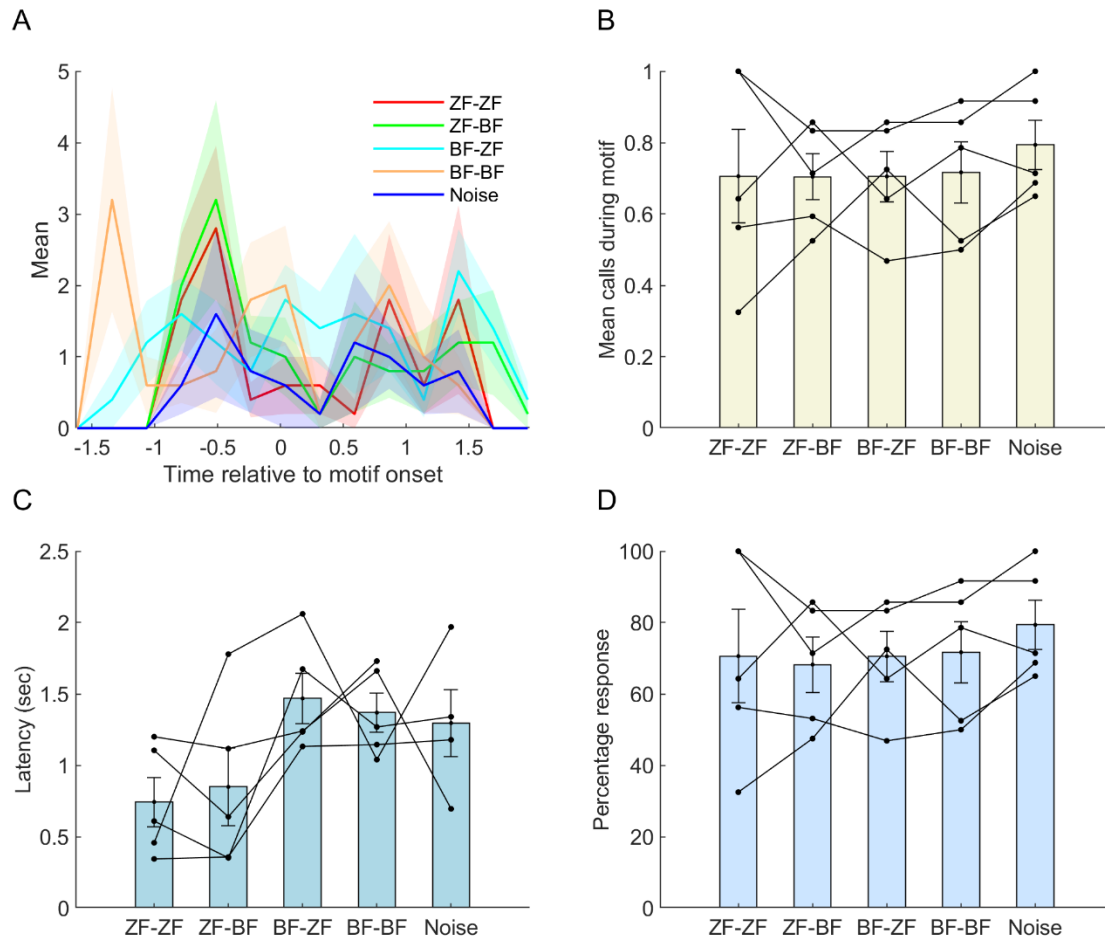


Figure A9. The presence of conspecific or heterospecific IN does not influence the female's response

A. Mean number of calls across all females (n = 5). Different colors represent different stimuli. Red: zebra finch song (ZF-ZF), Green: bengalese finch song starting with zebra finch IN (ZF-BF), Cyan: bengalese finch song starting with zebra finch IN (BF-ZF), Orange: bengalese finch song (BF-BF), and Blue: noise. The shaded region represents standard error. B, C, D, Bar graph for mean number of calls produced during motif (B), Latency (C), percentage response (D). Each bar represents different stimuli. Error bars are standard errors. Each point represents a bird. The line connects the same bird.