Respiration Monitoring During Introductory Notes of Zebra Finch Song

A Thesis

Submitted to

Indian Institute of Science Education and Research Pune in partial fulfilment of the requirements for the BS-MS Dual Degree Programme

By

Rubna P R

Registration Number: 20161114

Indian Institute of Science Education and Research, Pune

Dr. Homi Bhabha Road,

Pashan, Pune 411008, INDIA.

April, 2023

Supervisor: Raghav Rajan

Certificate

This is to certify that this dissertation entitled **Respiration Monitoring During Introductory Notes of Zebra Finch Song** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by **Ms Rubna P R** at Indian Institute of Science Education and Research under the supervision of **Dr Raghav Rajan**, Assistant Professor, Department of Biology, during the academic year 2022-2023.

Dr Raghav Rajan
Assistant Professor
Dept of Biology

Committee:

Dr Raghav Rajan
Dr Collins Assisi

This thesis is dedicated to Javed.

Declaration

I hereby declare that the matter embodied in the report entitled **Respiration Monitoring During Introductory Notes of Zebra Finch Song** are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Pune, under the supervision of Dr Raghav Rajan and the same has not been submitted elsewhere for any other degree.

Rubna P R
20 March 2023

Contents

Abstract

Thoracic air sac pressure of zebra finches was recorded during the song to study the correlation between respiration and introductory notes (INs) of their song. Introductory notes are short vocalizations preceding the song of the bird. They are thought to be part of motor preparatory activity happening in the brain before the song. But this hypothesis has been challenged by the existence of birds without Introductory notes and the fact that their number and acoustic structure of them can be influenced by learning. Here we try to see if motor preparation is done through respiratory modulations regardless of INs.

Acknowledgments

My deepest gratitude to my supervisor, Dr. Raghav Rajan, for the opportunity to conduct this study. His lectures were a great influence on me to get introduced to neuroethology and the discussions we had throughout the project helped me to become keener in my critical thinking. Thanks to him for helping me with a lot of steps in the instrumentation and making all the MATLAB scripts we needed for analysis. Thanks to Raghav and all my lab members who created a healthy workspace and made this journey easy and fun. Thanks to Dr.Collins Assisi for agreeing to be in TAC and giving valuable suggestions during my mid-year evaluation.

Thanks to Shikha Kalra who was an equal collaborator in the standardization of air sac surgery and gave inputs at multiple stages of my work. This study was started by Shivam Chitnis, who made the printed circuit boards I used at the beginning of the project and had already built a framework for the instrumentation part. He along with Sanjana Joshi, Divya Rao, and Shikha Kalra had laid out a foundation for the air sac cannulation surgeries with detailed notes and video recordings of the procedure. Shikha Kalra trained me for the surgery and post-surgery bird care after which together we troubleshot and improved the procedure.

Thanks to Lena Veit who gave suggestions to improve the cannulation step of the surgery and thanks to Amit Bhat and PerkinElmer facility, IISER Pune for the post-surgery C T scan images of birds.

Thanks to all my colleagues especially Ninad Thulkar, Sonam Chorol, Nandu T S, Anand Krishnan, and Dhanya Raj who helped me handle birds at different stages of the experiment. I want to mention Ninad's help in making crucial components for the recording setup and thank Shouvik Mandal and Darshan Dhanajkar for their time when they helped me with post-surgery bird care.

I want to acknowledge our funding sources IA/S/21/1/505621, CRG/2021/004690, and DST/CSRI/2017/163 for this project.

Thanking my parents and friends - Sonam Chorol, Dhanya Raj, and Titir Roy whose support has helped me throughout this work. Finally, Javed, thanks for being there, listening to all my day-to-day work troubles, helping me think more critically, and being my emotional islet, especially during the final stages of this work. The extra-long thank you note has to end with your name.

List of figures

List of tables

Contributions

Contributor name	Contributor role
Rubna P R, Raghav Rajan	Conceptualization Ideas
Rubna P R, Shikha Kalra, Shivam Chitnis Sanjana Joshi, Divya Rao	Methodology
Raghav Rajan	Software
Rubna P R, Raghav Rajan, Shikha Kalra	Validation
Rubna P R, Raghav Rajan	Formal analysis
Rubna P R, Shikha Kalra	Investigation
Raghav Rajan	Resources
Rubna P R, Raghav Rajan	Data Curation
Rubna P R	Writing - original draft preparation
Raghav Rajan, Rubna P R	Writing - review, and editing
Rubna P R, Raghav Rajan	Visualization
Raghav Rajan	Supervision
Raghav Rajan	Project administration
Raghav Rajan	Funding acquisition

Introduction

Before every voluntary movement, there is thought to be a measurable time interval where the brain prepares for the upcoming motor action using distinct neuronal activity patterns(Day *et al.*, 1989; Ghez *et al.*, 1991). This pre-movement neuronal activity is generally called motor preparation or motor planning and is characterized by stereotypical modulations in the firing patterns at many levels of the motor system, including, the pre-motor cortex and the motor cortex ((Churchland *et al.*, 2006). During motor preparation, the spatiotemporal dynamics of neural activity converges to a common state(Churchland *et al.*, 2006; Klein-Flügge *et al.*, 2013). It has been hypothesized the goal of motor preparation is to ensure that the brain reaches a ready state essential for the execution of a motor act irrespective of the initial conditions(Shenoy *et al.*, 2011; Svoboda and Li, 2018).

Defects in motor preparation can result in failure to initiate movements, faulty execution, or abnormal muscle spasms. It has been shown that neurodivergent conditions such as ASD and ADHD are accompanied by slow or deficient motor planning (Athanasiadou *et al.*, 2020). Also, disrupting preparatory activity in the brain delays movement initiation. So, understanding motor preparation would help us to get insights into failure and inaccuracy in motor performance, which has already been shown to occur in Parkinson's disease, speech apraxia, and dystonia (Terband *et al.*, 2009; Konczak and Abbruzzese, 2013; Marinelli *et al.*, 2017).

Preparatory neural activities have been explored and characterized mostly in humans and monkeys. However, these studies were predominantly done in the context of cue-triggered or stimulated movements where subjects were instructed to perform simple tasks (Lara *et al.*, 2018). it is hard to study preparation for complex sequential movements since they might not be precisely repeated every time and the variations would be difficult to keep track of. The patterns of preparatory activity can change based on the context and kinematics of the future action. Since in animals, generally, the tasks they perform would be something they have been trained to do in the experimental setup it is still unclear how the brain prepares for naturally learned, self-

initiated movements including rapid sets of movements where the whole structure of the sequence must be planned before their initiation.

A great model for understanding naturally learned, self-initiated movements is the songbird or Oscines which have a wide range of vocalizations and incredible control of the syringeal (vocal organ) muscle. Some species of songbirds produce songs that are learned vocalizations with temporal rhythm. These songs have sequential structures and require rapid action of the vocal muscles controlled by a group of functionally related brain areas (Elemans *et al.*, 2008; Fee and Scharff, 2010) making them ideal to study motor preparation of relatively complex movements.

Birdsong shares many features with speech and other learned motor sequences of humans. Birds learn to sing very much like how human babies learn to speak, by forming auditory memories of adult vocalizations. They have a critical period for vocal learning like humans with enhanced learning ability and a transition phase before producing a polished adult song. This transition phase is similar to the babbling of human babies (Doupe and Kuhl, 1998). These parallels make songbirds a potential system to understand speech motor patterns too. They have analogous forebrain areas with functional hierarchy and specialization suggesting similar neural mechanisms (Zhang *et al.*, 2023).

Among songbirds, the Zebra finch (*Taeniopygia guttata, Taeniopygia castanotis*) is a well-studied species and has provided a better understanding of the song motor program since they have highly stereotyped song features making it naturally reproducible each time the bird sings (Fee and Scharff, 2010). The song is a crucial part of the courtship ritual of the species and is learned from a conspecific tutor by male birds between 30 to 90 days after hatching (Price,1979; Zann 1996). The song consists of a series of syllables (vocal outputs) alternated with silent intervals. The repeating unit of the song is called a motif which consists of multiple syllables in a particular order. Though the motif is a stereotyped sequence of syllables, variability in this sequence is common and each syllable transition can have a range of probabilities associated with them (not in the zebra finch).

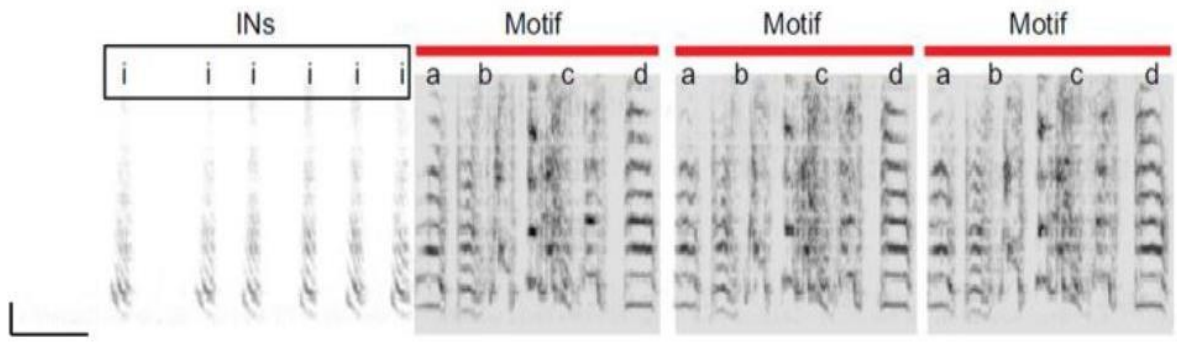

Fig.1 - A spectrogram of an adult male zebra finch song with 3 motifs (red bars) is shown. Syllables and INs are labelled with letters 'abcd' and 'i' respectively. The horizontal axis is time and the vertical axis represents frequency. Darkness of the spectrogram is a measure of sound amplitude. Scale bar: 200 ms (horizontal) and 1000 Hz (vertical).

Typically, songs are preceded by repeating short, vocalizations called introductory notes (INs). In a song bout, there can be a variable number of INs followed by multiple repeats of the motif with a silent gap or more INs between them (Fig.1) (Rao *et al.*, 2019). These singing events can be in the presence of a female bird along with other courtship displays or when the male bird is by himself with no audience. The former is called directed songs which are initiated by external stimuli and the latter, undirected songs which are self-initiated (Morris; Dunn and Zann, 1996). Having these two different contexts for the same learned motor sequence provides us with a great tool to learn preparatory mechanisms.

Preparation hypothesis of Introductory notes

As mentioned above, the zebra finch song almost always starts with a variable number of introductory notes (INs). It has been shown that intervals between successive INs decrease and the acoustic structure of INs become more stereotyped close to the song motif (Rajan and Doupe, 2013). A greater number of INs are produced before the song if the initial IN is acoustically more different from the final IN and this is typical in directed singing. The temporal progression of INs to the song has correlations with the neural activity in song motor areas of the brain where it reaches a stereotyped configuration before the song across renditions (Rajan and Doupe, 2013). This behavioral and neural correspondence has been a reason to think that INs are associated with motor preparation where both neural and vocal properties converge

on a common ready state from different initial conditions before initiating the motif. This is believed to be similar to the neuronal motor preparation seen in primates before simple movements.

This preparation hypothesis can also explain the greater number of INs produced during directed songs. The presence of the female could cause the internal neural dynamics to fall on an initial state farther from the ready state making the progression to song slower resulting in a higher number of INs (Rajan and Doupe, 2013) (Physiological changes such as increased heart rate have been reported during directed singing conditions). According to this, INs act like discrete steps to reach an acoustic endpoint which seems to be crucial for the upcoming song.

A song motif requires more INs if it is sung after a long silent interval. However, the number of INs does not correlate with the number of motifs sung after it or the length of the entire song bout ((Rajan and Doupe, 2013). Also, the motifs within the song bout do not necessarily have INs preceding them. Despite this variability in number, for a bird, acoustic properties are similar for INs at the same distance from the beginning of a motif and this similarity reaches the maximum for the final IN before a motif. The acoustic similarity between adjacent INs also increases closer to the motif (Rajan and Doupe, 2013; Rao *et al.*, 2019). These observations suggest IN progression and motif initiation can be the results of 2 independent processes (since INs are not obligatory for motif initiation all the time) but the former could be facilitating or acting like a switch for the repeated activation of the latter.

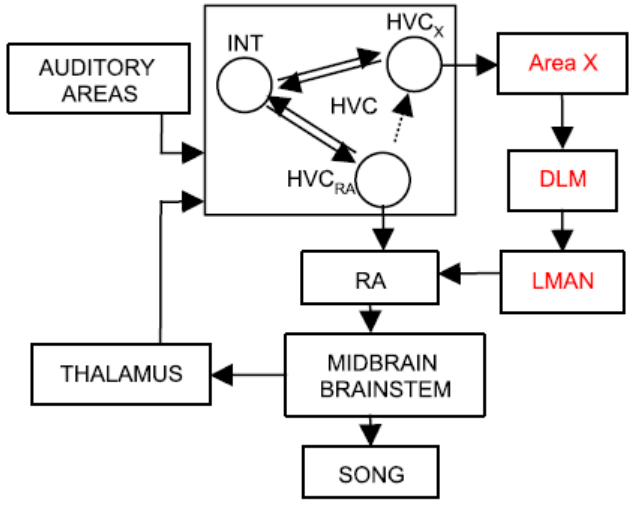

Fig.2 - Schematic of the song motor program is shown. The pre-motor nucleus HVC has 3 types of interconnected neurons – HVC_{RA} and HVC_X projection neurons, and interneurons. (Rajan and Doupe, 2013)

The pre-motor nucleus HVC of the zebra finch has distinct activity patterns during INs which become more stereotyped towards the last IN and reaches a significantly different state from all the earlier INs for the last IN. HVC is a critical brain area in the song motor program and its activity is required for normal song production (Aronov et al., 2008; Nottebohm et al., 1976). It has been shown that HVC has control over the temporal pattern of song sequences (Fee *et al.*, 2004; Long and Fee, 2008), but its role in song motor preparation has only been recently explored. The signature activity pattern of HVC neurons during the last IN is thought to provide a signal for song initiation to area X, the song basal ganglia (Fig.2) (Rajan and Doupe, 2013). So, the repeated introductory notes could be to carry the neural dynamics to the activation of downstream pathways in the song motor system.

Caveats of preparation Hypothesis

The preparation hypothesis offers a solid model for song motor preparation and explains why there are introductory notes before the song. This reasoning makes INs part of an innate mechanism for the execution of a learned motor sequence and the variation in its number and acoustic structure is governed by physiology and behavioral contexts. However, it has been shown that INs can be influenced by learning (Kalra *et al.*, 2021). The mean number and acoustic structure of INs produced by a bird are highly correlated with that of their tutor's INs. Learning of these two aspects of INs happens independent of song learning and the tutor does not have to be genetically related to the bird. The fact that the features of INs, a bird will produce, can be directed by the choice of tutoring challenges the idea that they are part of motor preparation and controlled by the preparatory load before song initiation.

Besides, some birds successfully produce songs without any introductory notes and the song premotor nucleus HVC of one such bird was observed to have 'IN–like' activity before the song (Rajan, unpublished data). This questions the role of introductory vocalizations in the transition from movement preparation to movement initiation. It is already established that neural activity changes occur in HVC much before INs which seems to be preparatory(Rajan, 2018; Daliparthi *et al.*, 2019) and

the origin(s) of these activity changes are not well understood. These activity patterns need not be present during INs and stronger and earlier such changes are correlated with more success in song initiation. This result with the fact that INs are not obligatory for motifs to happen every time indicates song motor preparation could be a combination of different networks and mechanisms not just stereotyped transitions in pre-bout neural activity of HVC.

On the other hand, it was observed that birds who were untutored or got tutored by songs without INs still produced an average number of 3 INs before their song (Kalra et al., 2021). This is explained by biological predispositions in IN production coming into action independent of learning. This makes one wonder about the evolutionary origins of such predispositions, one of which could be the functional relevance of INs in the motor pathway leading us back to the conundrum of IN-less birds.

So, it's evident that we still need more understanding of song motor functioning and its preparation. Here we try to revisit the preparatory hypothesis by looking at the respiratory patterns associated with INs. The motivation for this is respiration being another motor correlate closely associated with pre-song activity in HVC and might have preparatory functions which help to bypass the production of INs as in the case of IN-less birds.

More reasons to Look at respiration

Vocal production in songbirds is driven by the coordinated activity of the respiratory system along with the syrinx, the vocal organ (Schmidt and Martin Wild, 2014). It is the contraction of respiratory muscles that creates airflow through the trachea while the syringeal muscles regulate the resistance in this path, resulting in different vocalizations. Looking at aspects of respiration such as air-sac pressure, tracheal airflow, and respiratory muscle activity has helped to understand the song motor pattern better. The respiratory patterns during phonation are very different from quiet breathing (Franz and Goller, 2003). They can be distinguished by their significantly high amplitude in pressure fluctuations.

In the adult zebra finch, a correspondence between temporal and acoustic features of song, and respiratory pulses have been seen (Schmidt and Goller, 2016). The syllables of the song are associated with distinct expirations and the silent gaps between them are short, deep inspirations called mini-breaths (Fig.3) (Veit *et al.*, 2011). Expiratory and inspiratory pulses are stereotyped just like the song with characteristic patterns for each syllable of the song motif

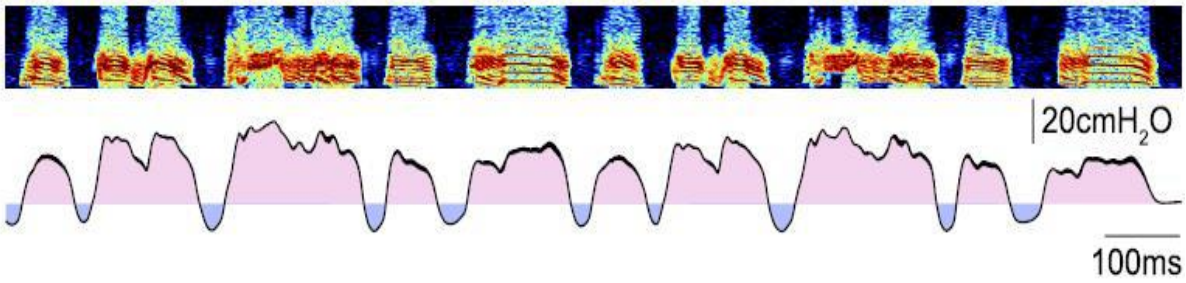

Fig.3 - Recording of thoracic air-sac pressure is shown along with corresponding vocalizations of an adult zebra finch. Pink-colored regions are expirations and blue are inspirations. The correlation between respiration and syllable duration can be seen here (Veit et al., 2011).

Studies on zebra finch and other songbirds have also shown an increase in respiratory tempo before the song starts (Méndez *et al. 2023*; Franz and Goller, 2003). It has been suggested that this faster breathing could be related to motor preparation for the song. Since respiratory pulse during the song is correlated with syllable morphology, looking for a similar correlation of respiration during INs could be interesting in the context of motor preparation. As seen in Fig.2, downstream neural connection after HVC and RA is going to the vocal muscles as well as respiratory muscles. Since INs are the vocalizations associated with motor preparation, it is expected to see such preparatory changes in respiration.

Hypotheses and proposals

- Distinct respiratory patterns are produced before motif initiation.

if respiration is involved in motor preparation, we expect to see distinct respiratory pulses before the song initiation. The pressure amplitude and temporal pattern of these will be more stereotyped close to the motif.

- Correlation of respiratory patterns with introductory notes

Since INs are vocalizations, it is expected to see some degree of correlation between them and respiratory pulses. However, whether there is a correspondence between each IN and an expiratory pulse is something to verify. For the adult zebra finch song, each expiration corresponds to a syllable and the silent gaps are filled with deep inspirations. We do not expect to see such a strong correlation in the temporal sequence of INs and respiration because gaps between introductory notes are much longer than the gaps between song syllables. These gaps could also include silent expirations. But we do predict the respiratory pulses to be more aligned with INs moving close to the song as a preparation for the motif, resulting in an increased tempo in the cycles.

- Respiratory pulses before the song do not correlate perfectly with IN number.

Though it is expected to see higher amplitude expiratory pulses for each IN, all such expirations do not have to be accompanied by an audible IN. In birds with no introductory notes, we predict IN-like (high-pressure amplitude) respiratory cycles or at least a pattern of breathing distinguishable from quiet respiration, present before the song.

- Presence of ~3 distinct respiratory cycles with or without INs.

A study done by Kalra et.al shows a tendency of birds to produce ~3 INs before their song even when their tutors had < 3 or > 3 Ins (Kalra *et al.,* 2021) Birds generally produce ~3, 60 ms long introductory notes on average, irrespective of how they were tutored as juveniles. Birds who were isolated and did not have any exposure to song or INs also produced an average number of ~3 INs. This has been explained by the biological predisposition of INs and could imply ~3 INs as optimal for motor preparation. In line with this study, we can expect ~3 respiratory cycles probably the last 3 respiratory cycles before song being crucial in motor preparation. In the case of birds with many introductory notes (5-7 0r more), it is possible to have the last 3 respiratory cycles more stereotyped and more aligned with INs. When the bird has few or no INs (0-2), we still predict that there will be ~3 stereotyped respiratory pulses with distinct properties.

These hypotheses can be verified by looking at the respiratory cycles (pressure recordings from air-sac) of birds before the song (during the INs) and analyzing their properties quantitatively.

Objectives

- Record sub-syringeal air-sac pressure of adult zebra finch before the song (during the INs) and visualize their respiratory patterns.
- Characterize the correlation between the acoustic and temporal patterns of INs and respiratory cycles.
- Analyze the respiratory pressure pulses before song initiation quantitatively (amplitude and tempo) and check how they are related to the IN number with a special interest in birds without INs.

Overall, in the context of this study, we would be able to critically review the preparation hypothesis of INs and find out how much role respiration has in motor preparation.

Materials and Methods

All the procedures were approved by The Institute of Animal Ethics Committee (IAEC), IISER Pune, and CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), New Delhi.

Birds

Adult male zebra finches (> 90 days post-hatch) were the subjects of this study. However, standardization of air-sac cannulation was done using 19 female zebra finches. Later, the same procedure was done on 50 male zebra finches. Birds were either bred in or obtained from vendors outside. They were raised in the bird colony maintained by the lab and tutored either by playbacks or adult male birds during development. While experimenting, they were kept in a sound-attenuating box either with other birds or alone and were under a 14:10 h day-night cycle. Before surgery, it was made sure that they can sing by presenting a conspecific bird in front of them.

Surgery - Air Sac Cannulation

To monitor respiration, a Silastic tube needed to be surgically implanted in the caudal thoracic air sac of the birds while they were anesthetized. This tube was connected to a pressure-sensing circuit after the recovery period.

Anatomy

The air sacs are thin membranous structures connected to lungs, and comprise most of the volume of the respiratory system. They do not directly participate in significant gas exchange but act as bellows to ventilate the lungs. There are nine air sacs in birds that can be grouped into cranial and caudal (Mclelland, 1989; Maina, 2002). The cranial group consists of the paired cervical air sacs, the unpaired clavicular air sac, and the paired cranial thoracic air sacs (Mclelland, 1989). The caudal group consists of the paired caudal thoracic air sacs and paired abdominal air sacs. In birds, unlike in mammals, both inspiration and expiration are active processes that require muscular activity. With contraction of the inspiratory muscles, the internal volume of the thoracoabdominal cavity increases and pressure within the air sacs becomes negative relative to ambient atmospheric pressure, and air flows from the atmosphere into the air sacs (Piiper and Scheid, 1999). During expiration the expiratory muscles contract and air sac pressure becomes positive flowing air out of them. To record air sac pressure generally the caudal thoracic air sacs(Schmidt and Martin Wild, 2014) (marked infig.4) are punctured. The basic method for this is established by Franz and Goller 2002 and Hartley and Suthers 1989.

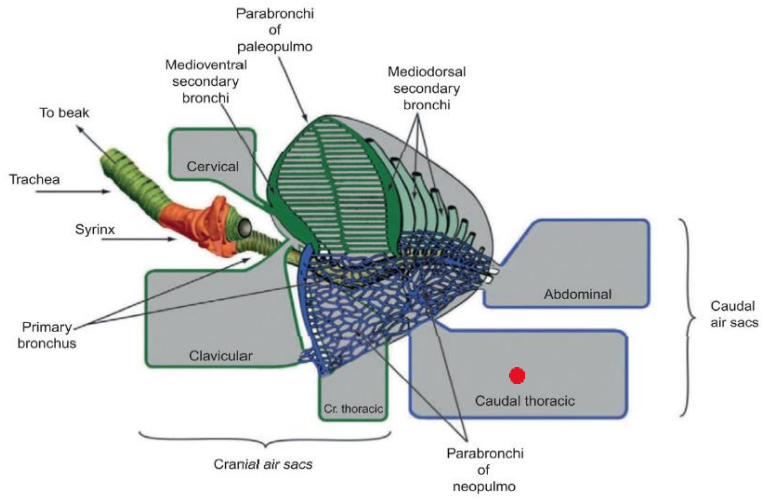

Fig.4 – Anatomy of the respiratory system of birds is shown. Lungs are connected to 3 pairs of (only one of them is shown out of a pair) air sacs and one unpaired (clavicular) air sac. Caudal thoracic air sac is marked (red dot). (Figure from Schmidt and Martin Wild, 2014)

Preparations

Implant construction

The tube to be implanted was made by inserting a longer (7 cm), thin silastic tube (inner diameter 1mm, outer diameter 2mm) into a shorter (5-7 mm), thick (inner diameter 2mm, outer diameter 3mm) one. The end with the thicker tube was to go into the air sac and its outer diameter was slightly more than the inter-costal distance of the last 2 ribs, at the site of the puncture made for implantation. This is to secure the implant within the rib cage by making it act like a barbed hook. The end with only the thinner tube was to be connected to a pressure sensor circuit later in the experiment.

Drugs and anesthesia

Birds were orally given an analgesic, meloxicam (0.25mg/kg) mixed with saline, and starved at least one hour before the surgery.

Right before the surgery, they were anesthetized using an induction box by supplying 4% of isofluorane in oxygen at a rate of 1L/min for 5 minutes. The duration of induction can vary based on how deep the birds get anesthetized. After induction birds were transferred to a surgery bed where a constant supply (1L/min) of isofluorane mixed with oxygen was given through a tube inserted into their mouth. The concentration of the isofluorane is kept between 0.5% and 3% at this stage but can be increased if the bird wakes up or responds during surgery. The bird is constantly monitored during surgery and isofluorane concentrations are adjusted based on how deep its breathing is. The supply was increased if the breathing is shallow and fast and it was reduced if the bird takes slow deep breaths.

Before making an incision for implantation of the tubing, a local anesthetic, Lignocaine (25ul) was injected subcutaneously at the site of the puncture to be and the skin was cut immediately after the injection. This was done by looking through a microscope and with special care not to get lidocaine into the blood of the bird as it is.

Surgery table and equipment

The surgery bed was made by taping a piece of foam on a clean table near the isofluorane supply. The major pieces of equipment were #5 forceps (2) and one micro scissor which were cleaned with alcohol and then sterilized using a glass bead sterilizer at 300°C. The tube to be implanted was also cleaned with 90% alcohol and

kept aside. A heat lamp was placed near the surgery bed since thermoregulation is impaired during anesthesia. Surgical sutures, tissue glue (Vetbond), cotton, saline, tissue papers, etc. were kept within reach (fig.4).

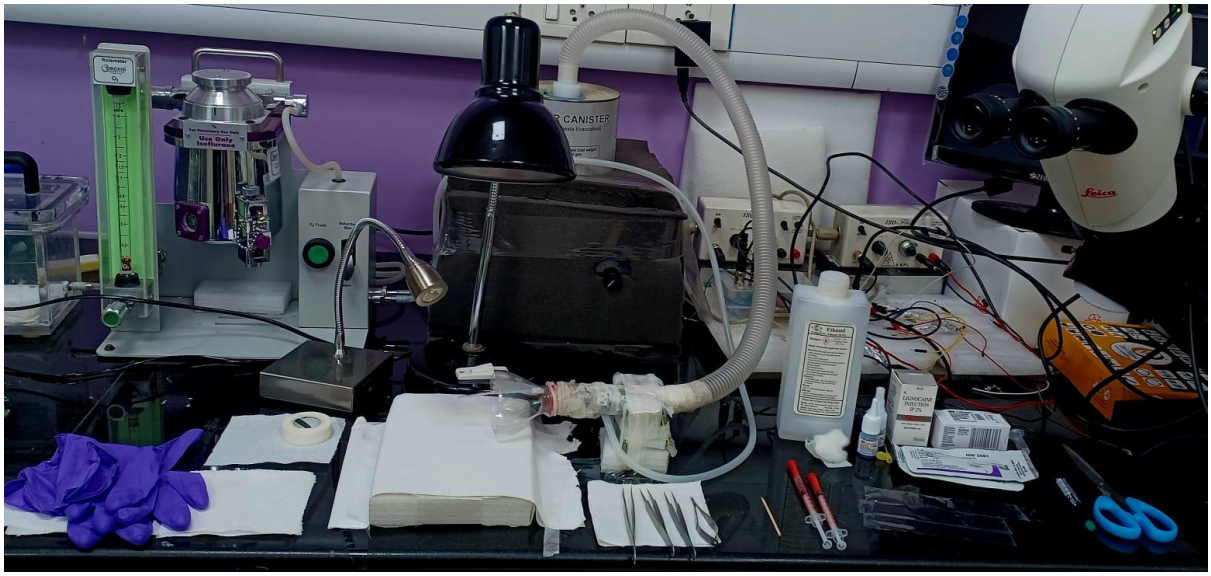

Fig.5 – Surgery table set up.

Implantation

The bird was taped to the surgery bed such that the left (or right) thoracic ribs are facing up. The feathers in the thoracic area are plucked using forceps. The skin is cleaned with alcohol and the last two ribs are located. 25 ul of Lidocaine is injected subcutaneously into this area with the help of a microscope immediately after which the skin is cut. Using a #5 forceps in the closed position a puncture is made between two ribs in such a way that only the narrow tip of the forceps goes inside. Open the forceps slowly keeping it inside and insert the tube with another forceps. Whether the tube went into the air sac can be checked by putting a small saline column at the other end of the tube which is supposed to pulsate with the pressure change. Depending on the amplitude of this pulse position of the tube can be adjusted. After this, it is sutured to one of the ribs using a surgical suture kit. Closing the wound, a tissue adhesive is applied around the tube, and the open end of it is closed using a piece of toothpick.

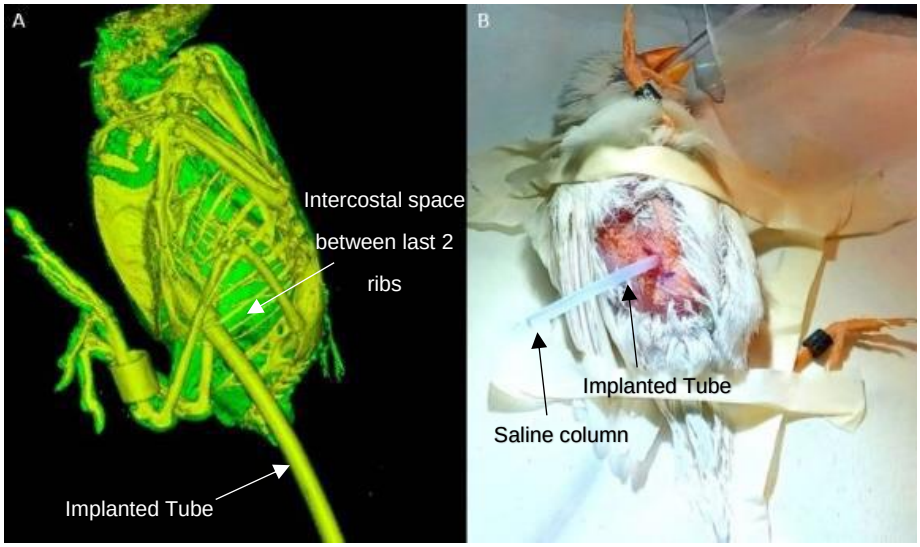

fig.5 - A: CT (Computed Tomography) image of male zebra finch after surgery. The tube with a larger diameter has an outer diameter close to the gap between the last 2 ribs. This arrangement makes sure the tube will not come out easily.
B: A female zebra finch, during surgery after cannulation of the air-sac. After implantation, a saline column is injected into the tube to see if it's pulsating, which means the tube went into the air sac. The amplitude of the pulse is often a measure of pressure signal strength.

Post-surgery and bird care

Post-surgery, it takes one to two days for the birds to recover. Waking up from anesthesia, it was made sure they ate properly. If not, 100ul dextrose was given orally. If the bird seemed sick the following day, meloxicam was given (0.25 mg/kg). The pressure sensor backpack was put on the bird after one day of surgery and the recording was started only when the birds were comfortable with the weight.

Pressure sensor backpack

The air sac pressure was recorded using a pressure-sensing circuit attached to the body of the bird like a backpack.

Gauge pressure sensor and circuit

The sensor was a gauge pressure transducer meaning it measured input pressure relative to the local atmospheric pressure and converted it to a proportional voltage signal. It worked based on the effect of piezoresistivity where the electrical resistance of the measuring component varies under mechanical stress.

The baseline voltage value, when nothing is connected to the sensor would correspond to ambient atmospheric pressure, and values below and above this baseline would represent sub-atmospheric and super-atmospheric pressures respectively. While connected to the implanted tube, voltage values below the baseline would occur while the bird breathes in (negative pressure) and voltage values above the baseline would be when the bird breathes out (positive pressure). So, the respiratory signal is expected to have cycles with troughs and crests during inspiration and expirations respectively.

The sensors used were FPM02PG, FHM02PG, and AG303 from Fujikura at different stages of the experiment (Supp.C). The signal was amplified by an external instrumentation amplifier (INA122P or INA 122U) made by Texas Instrumentation or an in-built amplifier in the sensor (for the pressure sensor AG303). The components of the circuit were soldered together using a normal circuit board (a board with equidistant holes) or a custom-made printed circuit board (PCB) or just to each other if the circuit had a minimal design. The circuit had to be connected to a 5V power supply and ground. So, a 3-pin was part of it which was used to connect the power supply, ground, and collect output voltage signal.

PCB design

PCBs used at different stages of the project were designed using Eagle Autodesk and then printed from PCB way / PCB Power (fig.6). Designs were made as compact as possible throughout the study and printed as thin flexible PCBs since the weight and size of the backpack make birds uncomfortable. Later, we switched to thin rigid PCBs as the flexible ones made the circuit less stable and non-functioning even under small mechanical stress. (Designs of all PCBs – Supp.D)

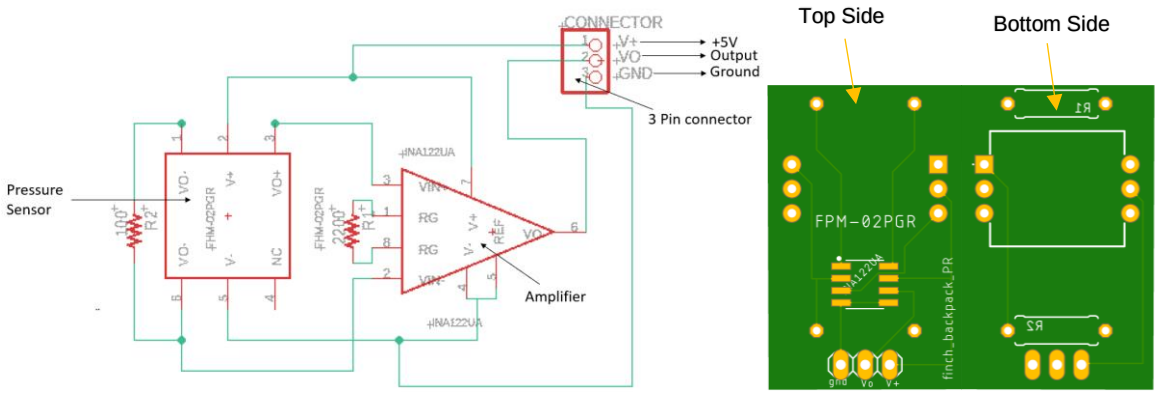

fig.6- Schematic of the A: pressure sensor circuit. B: PCB (FPM02PG(sensor), INA122U(amplifier))

Backpack Construction and attachment

Initially, the backpacks were made by soldering components onto normal circuit boards making connections with Cu wires and securing the connections by covering them with epoxy. Later we used PCBs to solder the components onto and covered the soldered portions with epoxy. Once it was dry, straps to put around the bird, made from elastic threads or rubber bands were attached. Different styles were tried at different stages since some birds kept removing the backpacks. Once the straps were put on the bird around its chest or wings, the backpack was secured by tying a thread along with it and putting a piece of tape around the knot to prevent birds from removing it.

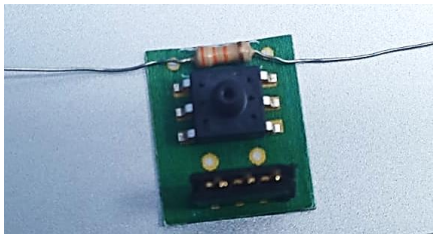

Fig 7. A pressure sensor backpack made using pressure sensor AG303.

Air sac pressure and sound recording

The pressure signal from the backpack and the vocalization of the birds had to be recorded simultaneously in synchrony to see the temporal correlations of respiration and introductory notes. This was done using a custom-written program called Okrank which started recording both signals in different channels by a single command.

Set-up

The recording was done in a sound box (NewTech Engineering Systems, 67 Bangalore, India), with the bird in its cage. The tube from the air sac of the bird was connected to the pressure sensor of the backpack and the 3pin ports of the backpack were connected to a commutator attached to the top of the cage. This tethering helped the bird to move freely in the cage. The 5V voltage supply and ground were connected to the commutator using an Arduino and the output voltage was connected to a DAQ (data acquisition) board connected to the computer.

An omnidirectional microphone (AKG68 517; Countryman Associates, Menlo Park, CA) was attached to the side wall of the cage, and the signal from it was amplified using a mixer and went into the same DAQ board.

Recording

Using Okrank, the pressure signal and sound were recorded in two different channels at the same time. Undirected recordings were done in the early mornings, and sometimes during the day, without any interference to the sound box or the bird. For directed recording, a live female bird was shown to the bird in each recording session. To get rid of the vocalization of the female bird, video recordings of female birds were used. Interestingly, sometimes the bird sang to an inanimate puppet of a male bird.

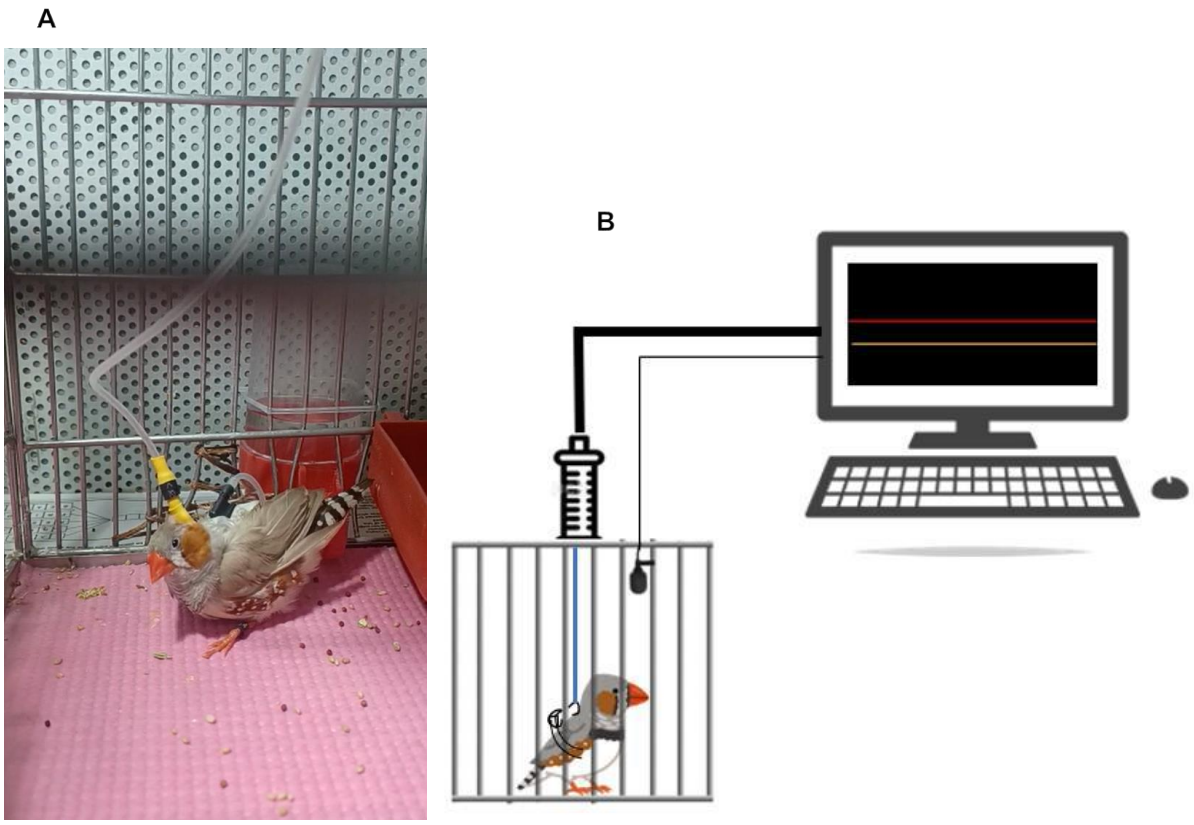

Fig.8 – A: Recording set up with bird. The tube going up from the bird has Cu wires inside it, and they are connected to a commutater.B: a schematic of the complete recording set up. The song is recorded through the mic and the respiratory pressure signal is collected to a DAQ board through a commutator.

Analysis

All analyses were done using custom-written scripts in MATLAB (Supp. E).

Song segmentation and labeling

Each Audio file contained sound between 1 and 4 KHz. They were segmented into syllables* based on a user-defined amplitude threshold and manually labeled using the MATLAB program ASSL (AutoSongSegmentLabel) (Fig.9). The boundaries of these segments were considered as the onset and offset time of each syllable. If there were a large set of files, a previously labeled file was used as a template, and syllables were labeled automatically using a template matching program and reviewed later. INs were identified as repeating vocalizations at the beginning of a song and song motifs were identified as the repeating unit (set of syllables) during an entire song event. Song motifs with a gap of 2 or more seconds between them were considered separate bouts. For the initial analysis, all the INs were labeled as ‘ i ’, and song syllables were labeled with lowercase alphabets (see Sup.E For raw data). For the second part of the analysis, INs were given different labels (‘i,j,k,l,m,n’) based on when they occur relative to the beginning of the motif (Calls of the male birds were represented by uppercase alphabets. Female calls got recorded during the directed song were either not labeled or labeled as ‘x’ and were not part of any kind of analysis. Any unknown vocalization during the directed or undirected song is also labeled as ‘x’ and was not considered in the analysis.

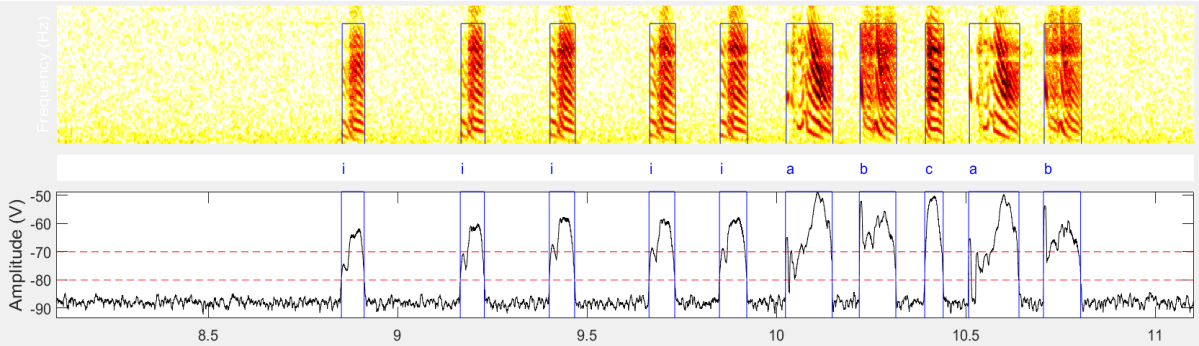

Fig.9 – How syllables were segmented based on amplitude threshold (using ASSL) is shown in the figure. INs and song syllables are labelled as ‘i’ and ‘a,b,c’ respectively. x axis represents time (Sec) and the y axis for the spectrogram represents frequency (Hz). The lower part of the y axis shows a measure of amplitude in voltage. As shown, syllables are marked using a common amplitude threshold

Segmentation of respiratory cycles

Recordings of the respiratory pressure signal contained noises that are thought to be electrical noise from the surroundings, physiological signals from the bird (like heartbeats or pulses), and noise from the pressure sensor circuit itself. Therefore, any voltage signal with 20 Hz or more frequency was filtered from these recordings and the pressure signal was smoothened (averaging data points at a rate of 3200/sec which is 1/10th of the sampling rate).

The filtered and smoothened data showed oscillatory patterns representing inspiratory and expiratory cycles. The baseline of these oscillations was considered to be at the mean pressure over the entire duration of each file (20 seconds). Whenever the respiration curve crosses this mean value we considered it as a transition between the expiratory and inspiratory phases (Fig.10). These time points were marked as the onset and offset of individual expiratory (and inspiratory) cycles and used in the analysis of temporal correlation with syllables.

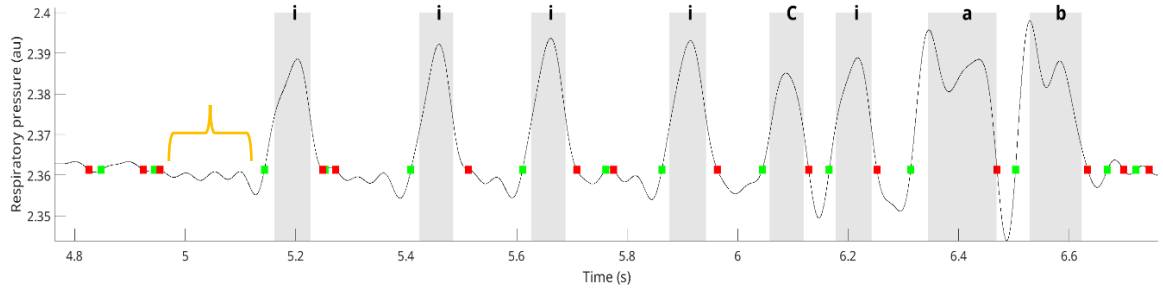

Fig.10 – Figure shows the segmentation of respiratory cycles during song. X axis represent time and y axis arrows voltage values proportional to air sac pressure (au stands for arbitrary unit). Grey patches represent syllable boundaries and INs and song syllables are labeled. The intercept of the pressure signal curve with a baseline marks onsets (green points) and offset (red points) of expiratory cycles. All these points are successfully marked for INs and song syllables. Regions where there are oscillatory patterns but no expiration onset-offset marked can be seen in the silent gaps (yellow

This identification of expiratory and inspiratory cycles was done using a custom-written program and as shown in Fig.10 and Fig.11 It did not mark many of the quiet respiratory cycles and sometimes wrongly marked onset-offset points of expirations during the song. This was due to poor signal during quiet respiration and fluctuations in the baseline pressure value around vocalizations. These errors in the segmentation of respiration were reflected in the results which we tried to reduce by eliminating syllables from the analysis if their onset (offset) and the onset (offset) of expiration

associated with them have more than a one-second gap. For INs this criteria for eliminating it from the analysis was having more than a 2-second gap between syllable onset (offset) and expiration onset (offset).

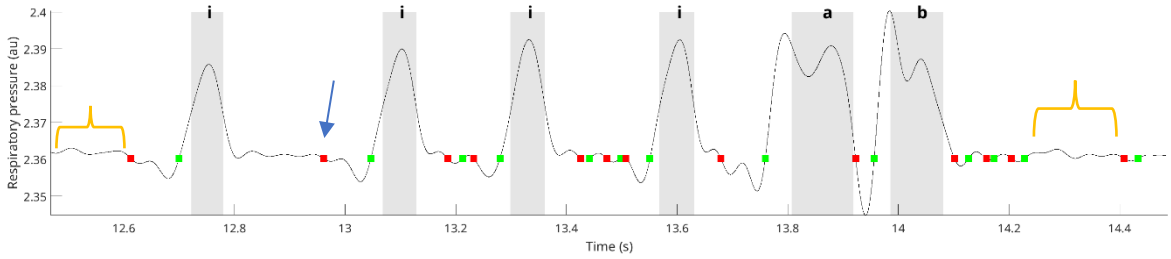

Fig.11 – Segmentation of respiratory cycles is shown where the program made errors in marking onsets and offsets of expirations. Silent gaps where EP cycles are not marked are shown by yellow brackets. An instance where the EP offset is wrongly marked is shown by blue arrow.

Results

Air sac surgery was done on 66 zebra finches of which 28 surgeries were on female birds that we used to standardize the procedure. After improving the survival rates, surgery was done on 38 male zebra finches of which 19 birds survived the implantation procedure. Out of these, 4 birds died or got the implanted tube removed before getting to the recording phase. 15 birds were subjected to sound and air sac pressure recording of which very few were vocally responsive with the pressure sensor backpack and connections to the commutator. After a considerable reduction in the weight of the backpack birds started to produce vocalizations and the songs of 4 birds were recorded along with air sac pressure. The analysis includes directed and undirected songs from 2 birds, only undirected songs from one bird, and only directed songs from another bird constituting 6 sets of song data. (See Supp. B - bird statistics)

Standardisation of Air sac cannulation

Air-sac cannulation has been standardized and the success rate of surgeries has been improved. However, a slight variation in the optimal angle(s) and depth of the puncturing of the body wall can still result in the death of the bird. Instead of just puncturing with the forceps and inserting the tube without seeing the location where the tube going, we were able to see the air sac membrane through a microscope and place the tube more accurately in the last few surgeries. This method did not guarantee

a 100 % success rate in surgeries, since the connective tissues of other vital organs can have a similar texture to the air sac membrane and accidentally hitting them has resulted in heavy bleeding and the death of birds.

To see why the pressure signal goes off in birds after one or two weeks, we dissected birds and found the improper insertion of the tube could be resulting in its dislocation from the air sac. This has helped us to understand air sac structure and anatomy better (see Supp. F - video of air sac beating in an anesthetized bird). To make surgeries successful all the time, we need a clear understanding of the location, orientation, size, and shape of the air sac relative to the last 2 ribs of the bird. By performing a post-mortem on birds, we were able to follow the anatomy of the respiratory system to the lungs and cranial air sacs but could not reach thoracic air sacs (Fig12).

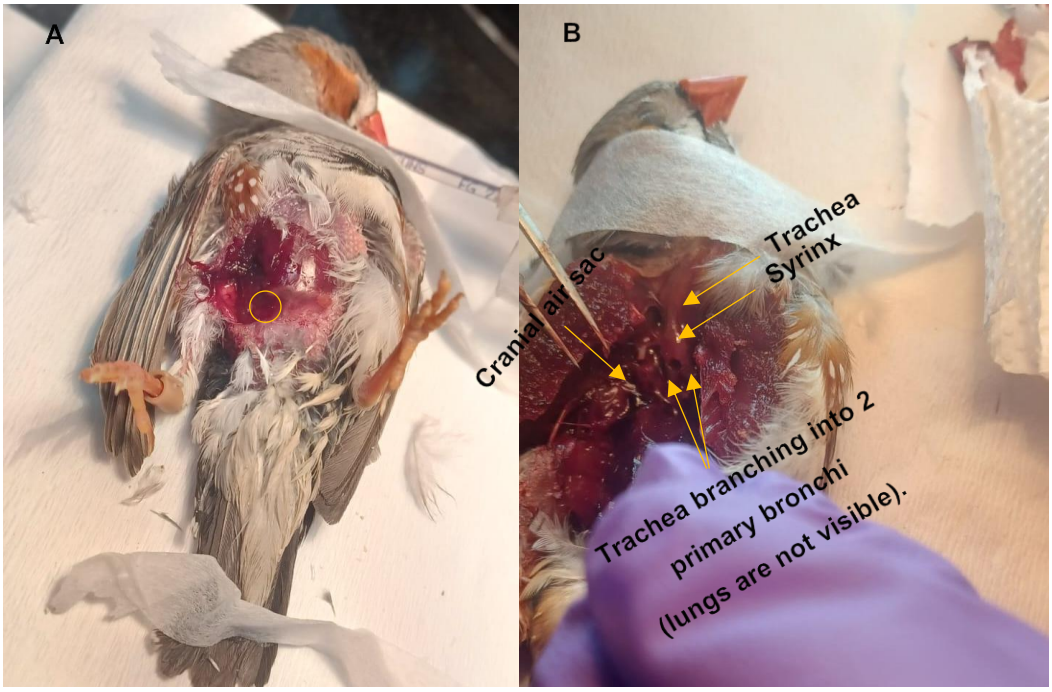

Fig.12- A: An anesthetised bird dissected in the thoracic area. A membranous beating structure was found there which we thought to be one of the caudal thoracic air sac (yellow circle). B: Post-mortem of a bird – entire chest is dissected. Trachea, Syrinx, and what we assumed to be a cranial air sac are marked. 2 primary bronchi and the lungs connected to them were seen during the procedure. Lungs are not visible in the photo.

Optimization of pressure sensor backpack

Birds were not singing while carrying the pressure sensor backpack. So, it was necessary to reduce its weight and size as much as possible. With the use of printed circuit boards, we managed to reduce the weight of backpacks from 4 g to almost 1 g. The weight alterations between different backpacks are tabulated (table 1). In the final experiment, the circuit components were soldered together without a PCB where we used the smallest pressure sensor available which had a built-in amplifier. (See Supp. C – datasheets of pressure sensors and amplifiers used)

Backpack (denoted by its components)	Final Weight (g)
FGM02PG + INA122P (normal circuit board)	4
FPM02PG + INA122U (Thin flexible PCB)	3.85
FHM02PG + INA 122U (Thin flexible PCB)	1.99
AG303 (Thin, rigid PCB)	1.2
AG303 (No PCB)	1.15

Table.1- Different pressure sensor backpacks used throughout the study (chronological order) are listed in column 1. Their final weight (after putting epoxy) is shown in column 2. Backpacks are denoted by the pressure sensor and amplifier used (in that order). The last 2 backpacks only had pressure sensors with built-in amplifiers.

Respiratory patterns during the song

Respiratory data during song syllables agrees with existing results

As already known, during syllables, the respiration of the birds is in the expiratory phase, and during the silent intervals, it is in the inspiratory phase. As expected, each syllable is produced during a single expiratory pulse and each silent gap is filled by a single inspiratory pulse called mini breaths (Fig.13). The inspiratory and expiratory pressures during syllables and mini breaths are significantly higher compared to quiet respirations. Just like the spectral features, the respiratory patterns during syllables are also stereotyped with characteristic shapes of expiratory pressure curves which are represented by the uniform color distribution of the heatmap (Fig.14).

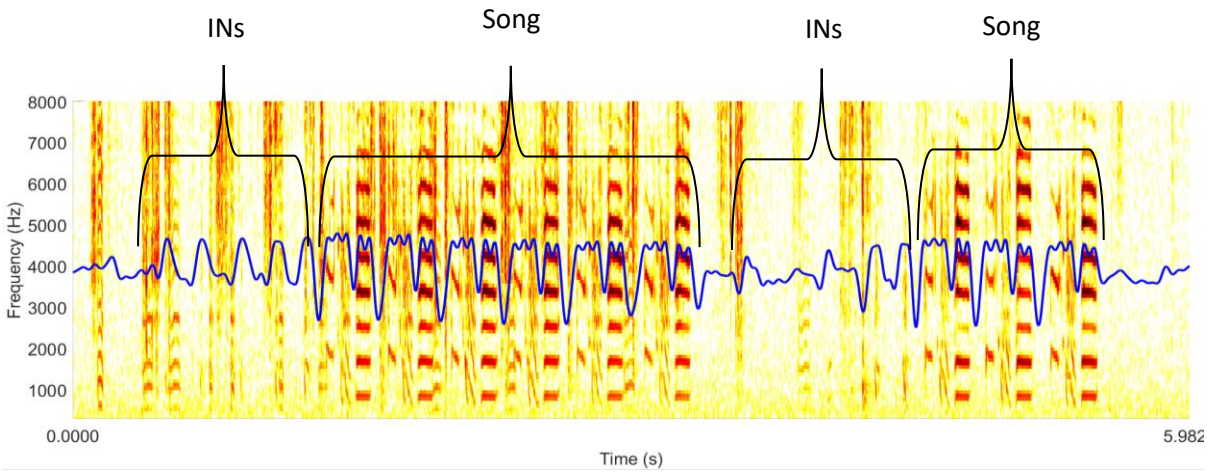

Fig.13 – Spectrogram of a zebra finch song overlayed with air-sac pressure (blue line) signal is shown in the figure. For the spectrogram x axis represents time, y axis is frequency and the darkness of the spectrum represents sound amplitude. For the pressure data, x axis represents time. The arbitrary units correspond to pressure is not shown but the increased amplitude of the signal is qualitatively represented. Song and INs are marked. Single higher amplitude expiratory pulses can be seen during each song syllable and short sharp inspiratory pulses called mini breaths fills the silent gaps between them.

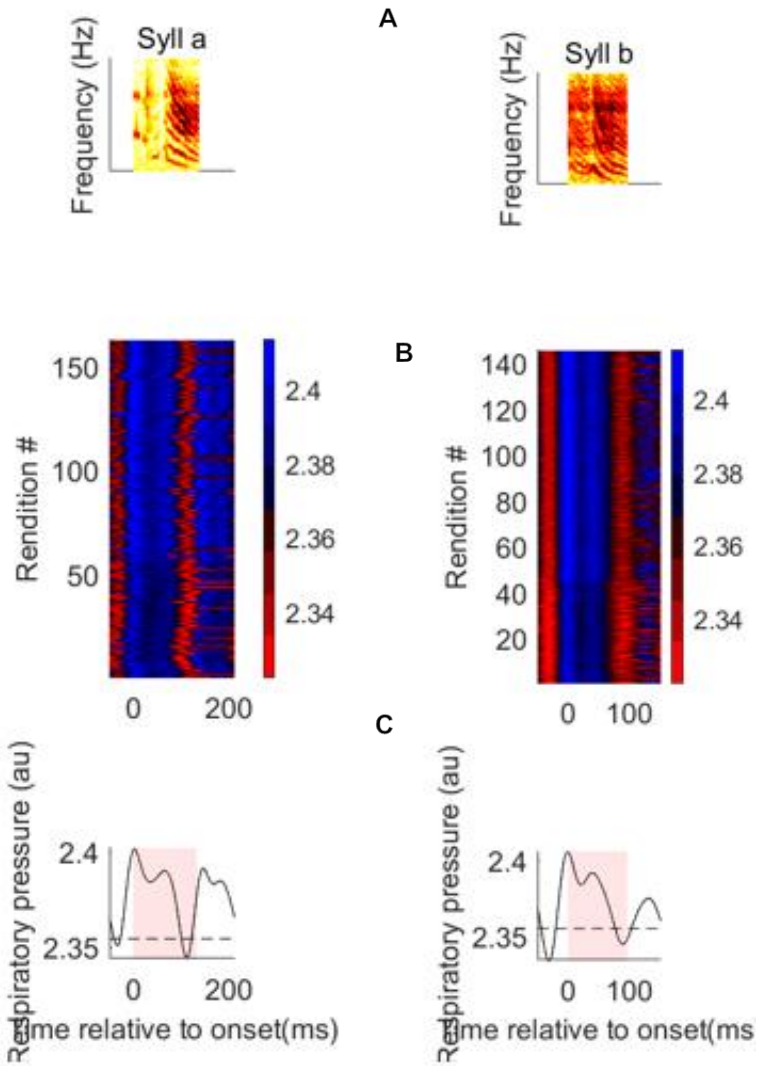

Fig.14 – Figure shows two syllables (a,b) from the song of the same bird with spectrograms at the top (A), average respiratory curves at the bottom (C).

The figures in the middle shows a heat map for each renditions of those syllables (B). Colours assigned for pressure values are shown along with them.

The red patches in C represent the boundaries of syllables averaged over all the renditions.

x axis of all the plots represent time. y axis of spectrograms shows sound frequency. y axis of heatmaps shows number of renditions of the syllables. In C, y axis shows pressure represented in arbitrary units.

Respiratory patterns during Introductory notes

For this part of the analysis, all the introductory notes were labeled as ‘i’ despite their distance from the song motif, and their average properties were compared to the average properties of different song syllables and calls.

Distinct expiratory pulses occur for each IN

Across birds, during directed and undirected singing, higher amplitude expiratory pulses were found to be associated with each introductory note (Fig.15). Such EP pulses were not found before INs, and based on the data from 4 birds, the number of INs and the number of these higher amplitude pulses are in one-to-one correlation.

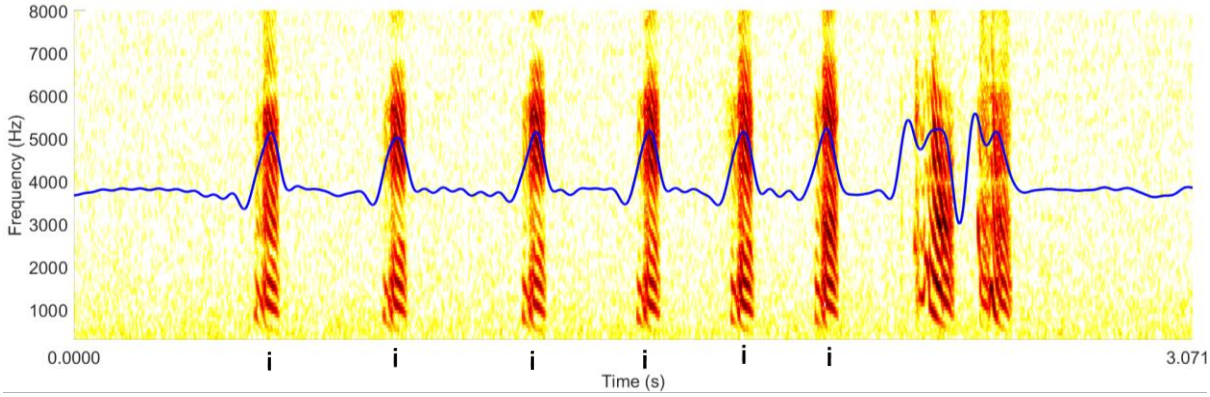

Fig.15 – Spectrogram of a zebra finch song overlayed with air-sac pressure (blue line) signal is shown. Only INs are labelled. The blue line represents the qualitative fluctuations in air sac pressure. Both spectrogram and the pressure signal share the same time axis.

The onset and Offset of INs and those of expiratory pulses are correlated

IN and EP Onsets

The time difference between the onset of each IN and the onset of the corresponding expiration was calculated as IN onset time - EP Onset time. This value being zero would mean a strong coupling between the initiation of INs and expiration. Positive values would indicate that INs start slightly after the onset of expirations. If INs start before the onset of expirations we would see negative values for this parameter. The

same time difference for song syllables and calls was also calculated and the distribution of these values across renditions was compared.

As seen in Fig.16 (blue) Onset differences are not strictly zero implying there's no tight correlation between IN and expiration onset. However, these values are close to zero for all vocalizations (INs, song syllables, and calls) with no significant differences across them.

Blue plots in Fig.16 show the distribution of Onset time difference between vocalizations and corresponding Eps for 2 different birds Top and bottom figures) in directed and undirected contexts (shown side by side). 3 out of 4 of these distributions show high variability for INs compared to the variability for song syllables and the call. However, considering the possible errors in assigning expiratory pulses to syllables (mentioned in the Analysis part of Methods) and less number of birds, it is not possible to conclude anything about this variability.

IN and EP Offsets

Similar to the Onset analysis, the time difference between the offset of each syllable and the corresponding EP was calculated. The offset time of syllables was subtracted from the offset time of Eps so that positive values would mean expirations ending after syllables. As seen in the onset analysis, these values were not strictly zero but close to zero for all the vocalizations (Fig.16 – red plots).

The distribution of these values shows no significant difference between INs, syllables, and calls. Same as the onset analysis distributions of INs have higher variability in most of the cases, which is only an observation, not something to be concluded because of fewer data.

Expirations wrap around vocalizations

For most of the vocalizations, both onset differences and offset differences are positive implying expirations start before syllables and end after syllables. However, this trend is very weak for the onsets of INs where the vocalizations can start before expirations. The offset of INs and corresponding expirations show a similar trend to that of song syllables and calls with expirations ending after vocalizations.

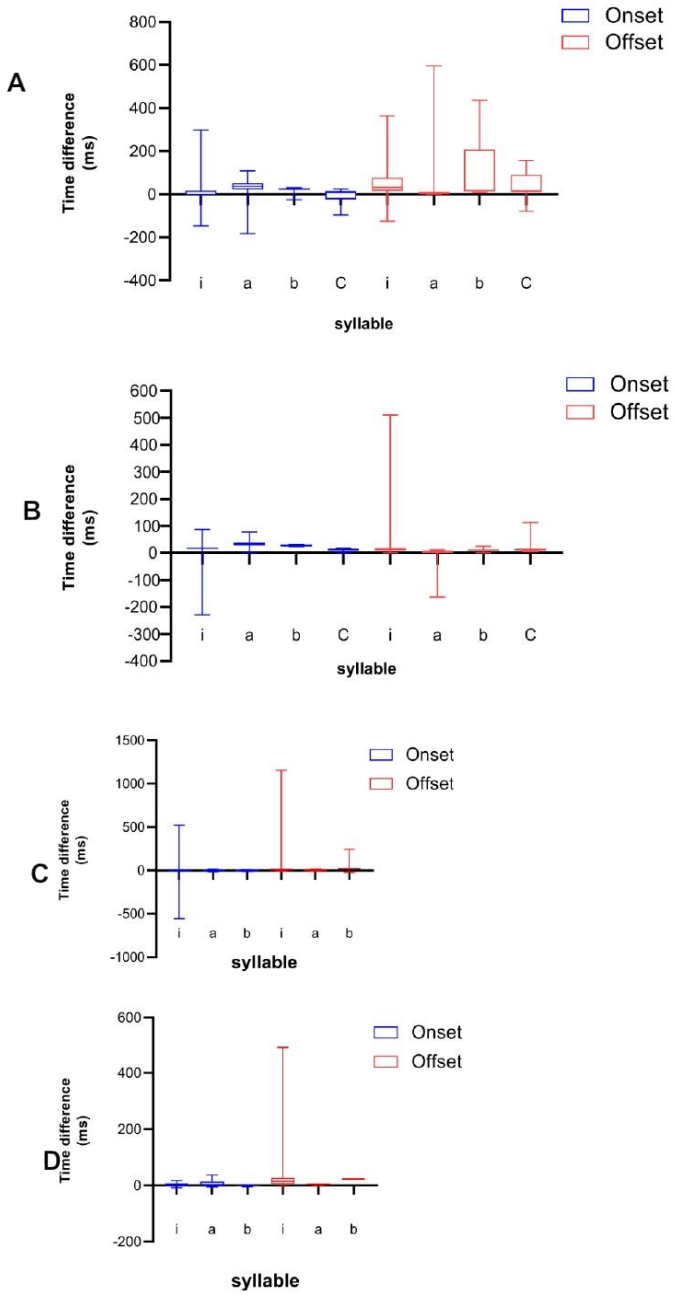

Fig.16 – Box plots showing temporal correlation of IN and song syllables with their expiration. Values on y axis are either syllable onset time subtracted from expiration onset time (for blue plots) or Expiration offset subtracted from syllable offset (for red plots).

A: Data from a single bird in directed condition.

B: Data from the same bird in A in undirected conditions.

C: Data from different bird in directed conditions.

D: data from the same bird as C in undirected conditions

X axis shows syllable labels. i for IN and a,b are song syllables. C is a long call.

Expiratory pressure does not have a significant difference between INs and song syllables.

Higher expiratory pressures during any vocalization were expected to see because of the sound production mechanism in adult birds. Average pressure during expirations associated with each IN was calculated. These values were compared with the average expiratory pressure associated with song syllables and a long call (Fig.17). Expiratory pressure values during INs were found to be comparable to that of other

vocalizations. The distribution of these values for INs does not show any pattern different from song syllables or calls.

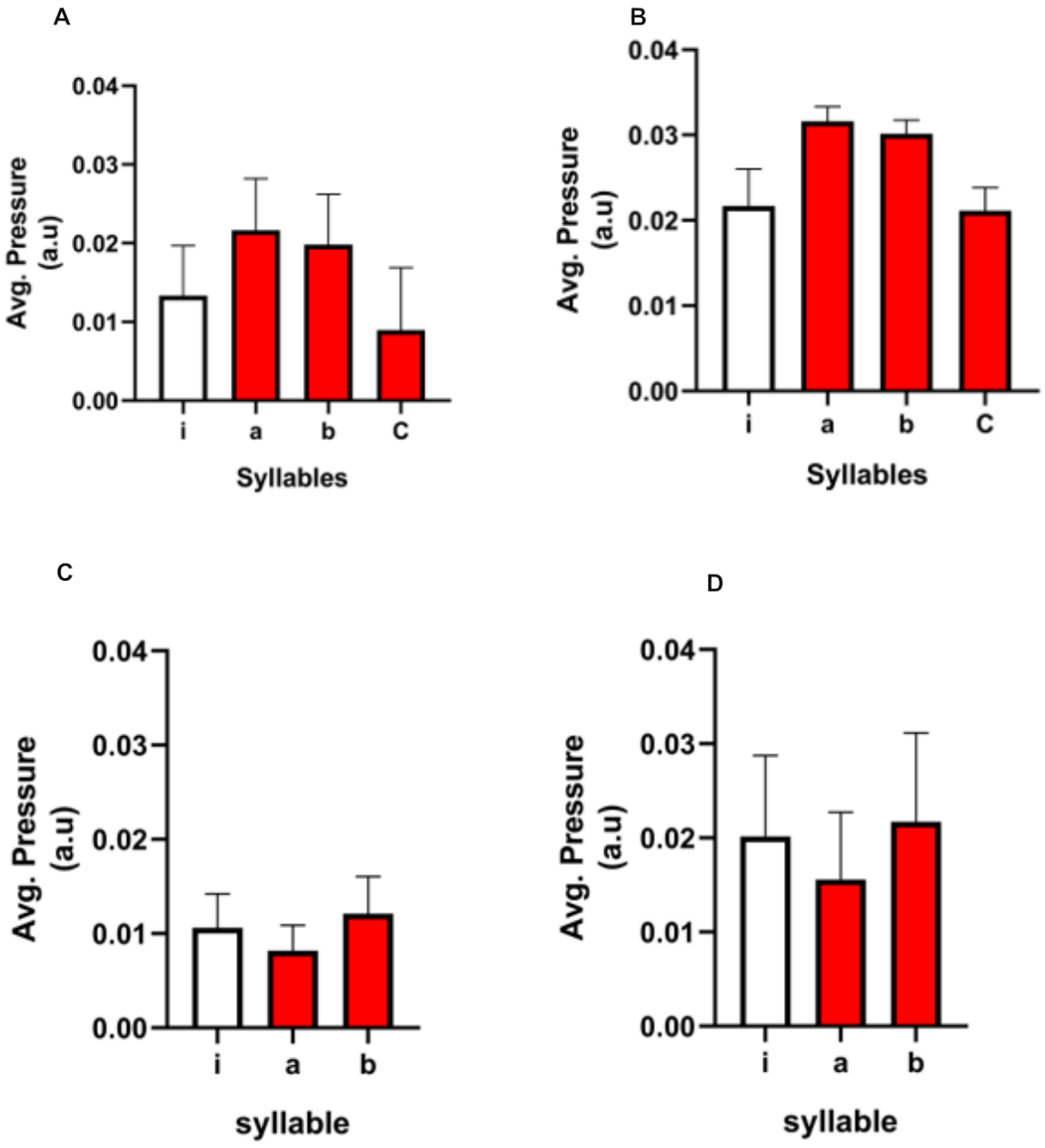

Fig.17 – Average expiratory pressure (in arbitrary units) during INs and song syllables are shown.

A, B represents a single bird in directed and undirected conditions respectively. C,D represents data from a different bird in directed and undirected conditions respectively. X axis shows syllable labels. a,b are song syllables, and C is a call (red). i (white) represents INs averaged across all song bouts of the bird in specified condition.

The fraction expiratory pulses occupied by syllables shows no significant difference across vocalizations.

Fig. 18 shows the average values for the fraction of each higher amplitude expiration occupied by corresponding vocalizations. It does not show any pattern for INs or any other kind of vocalizations. These values are close to 1 for the second bird in directed songs (Fig. 18C), but in that case, they are higher for all the vocalizations.

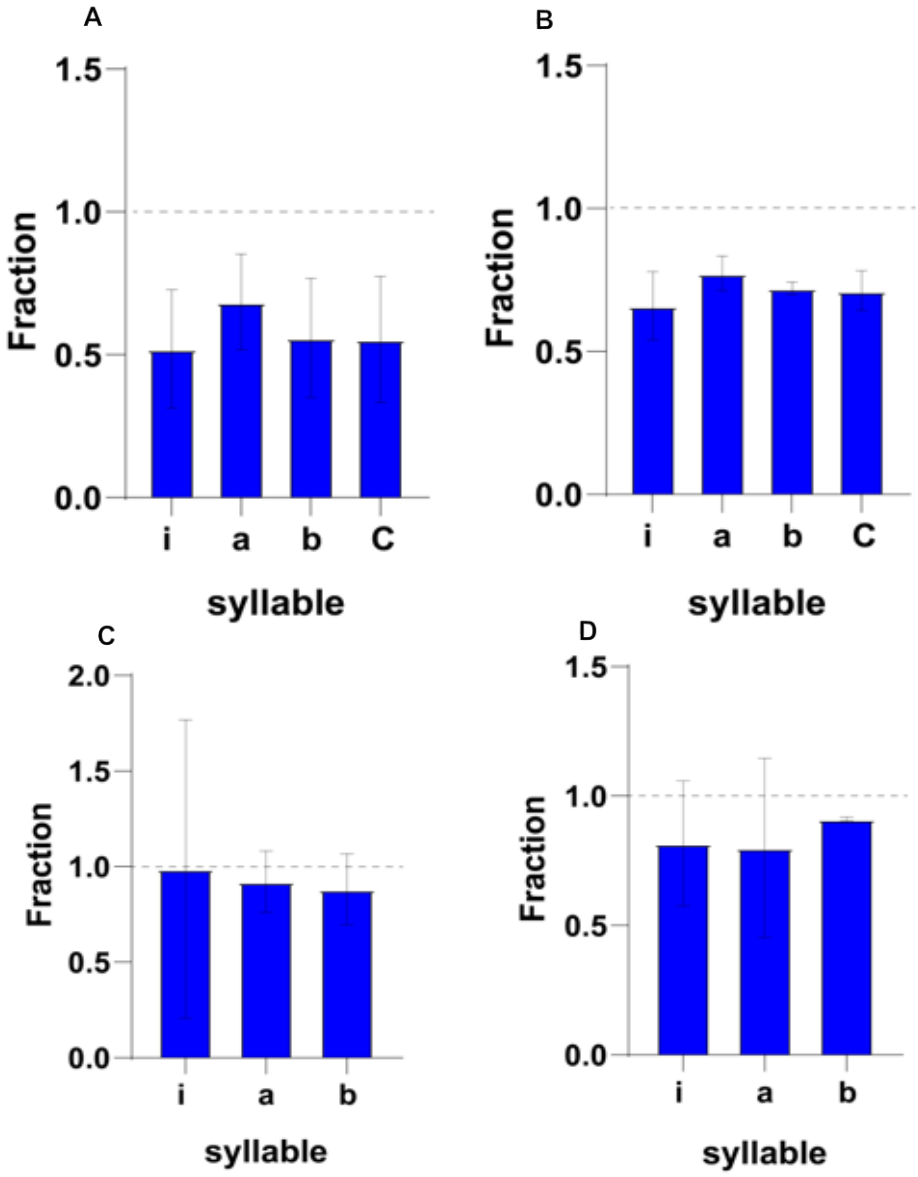

Fig.18 – Fraction of expiratory pulses occupied by vocalisations is shown for INs (i), song syllables (a,b) and call (C). A, B represents a single bird in directed and undirected conditions respectively. C,D represents data from a different bird in directed and undirected conditions respectively.

Changes in respiratory patterns toward the song motif.

Average values of different parameters, combining INs and their expirations did not show any strong pattern compared to other vocalizations. However, these properties were analyzed considering all the INs together as a group against each song syllable or call. A detailed analysis of the same properties across different INs would show what's happening as the bird gets close to the motif initiation.

For this part of the analysis, INs were labeled separately based on their distance from song motifs as i, j,k,l,m,n, etc with i being the last IN (closest to motif). Individual song bouts with different numbers of INs were manually picked out from the recordings with the elimination of any bout where calls occurred in between INs (occurrence calls would affect the consistency of IN numbering). Only a few song bouts were obtained satisfying this criterion, therefore analysis was done by comparing individual INs in a song bout rather than comparing average values of INs with the same index.

Temporal correlations with expiration across INs

Onset–offset analysis

Even though there are outliers, the onset and offset differences between INs and expirations converge to a common point close to the song motifs (Fig.19, Fig.20). For most of the INs, these values are positive, implying expirations starting before IN onset and ending after IN offset. When these parameters show negative values, they are for initial INs and there is a tendency to converge toward a positive common value closer to the motif. Values deviating from this general trend are from the same INs (* in Fig.19A, Fig.20A) in the onset and offset analysis indicating potential errors in the respiration segmentation (mentioned in methods).

The fraction of expiratory pulses occupied by vocalization shows no significant difference across INs.

This is similar to the result from IN vs song-syllable analysis. The function of higher amplitude expiratory pulses occupied by vocalization has no patterns going toward the motif (Fig.21). Whenever this fraction is higher for a bird it is higher for all the INs.

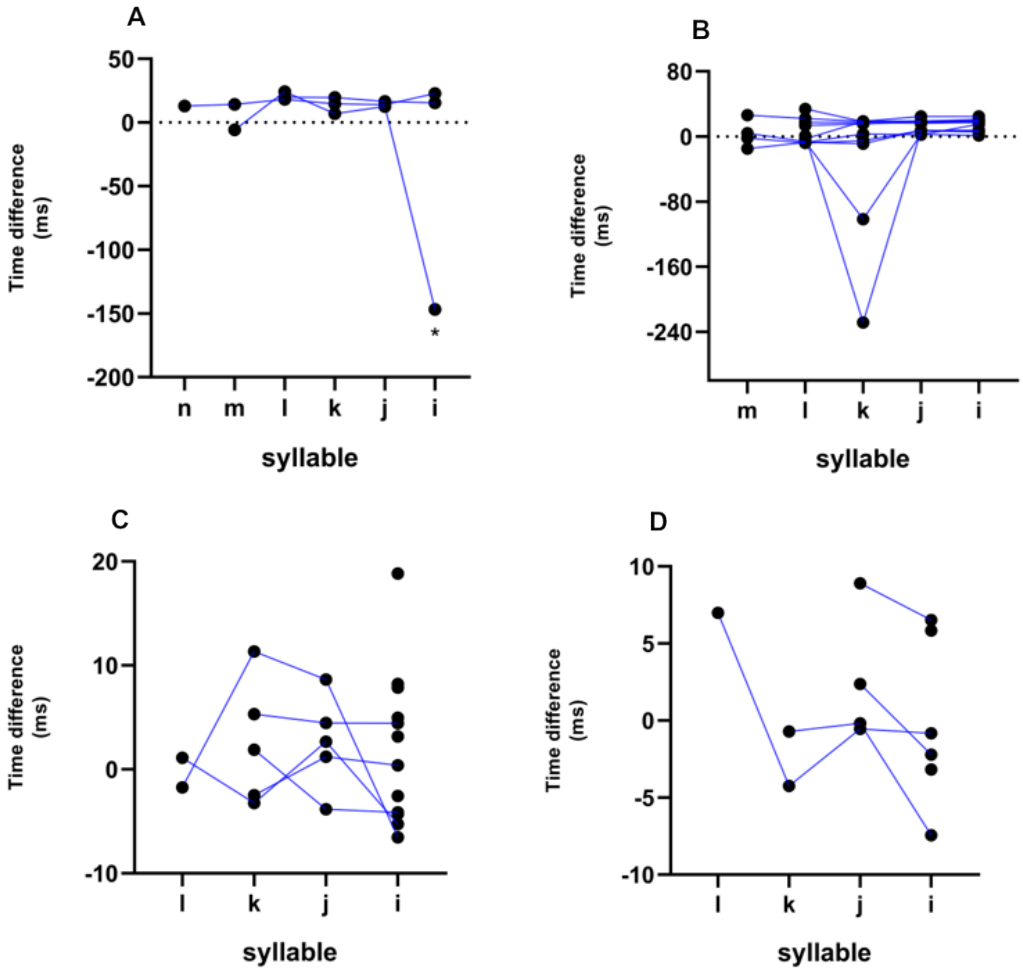

Fig.19 – Correlation of onset time of different INs with that of their expirations. Values on y axis are syllable onset time subtracted from expiration onset time. x axis shows syllable labels, i representing last IN, j for second to last IN, etc.

A: Data from a single bird in directed condition. B: Data from the same bird in A but undirected conditions. C: Data from different bird in directed conditions. D: data from the same bird as C but in undirected conditions

Expiratory pressure increases moving towards the last IN

This increase is in line with the known increase in sound amplitude toward the song motif. Since vocalization is associated with higher expiratory pressures than quiet expirations this increase in pressure amplitude with sound amplitude is not surprising. (Fig. 22). However, these values were increasing for each song bout independently without any convergence towards a common value.

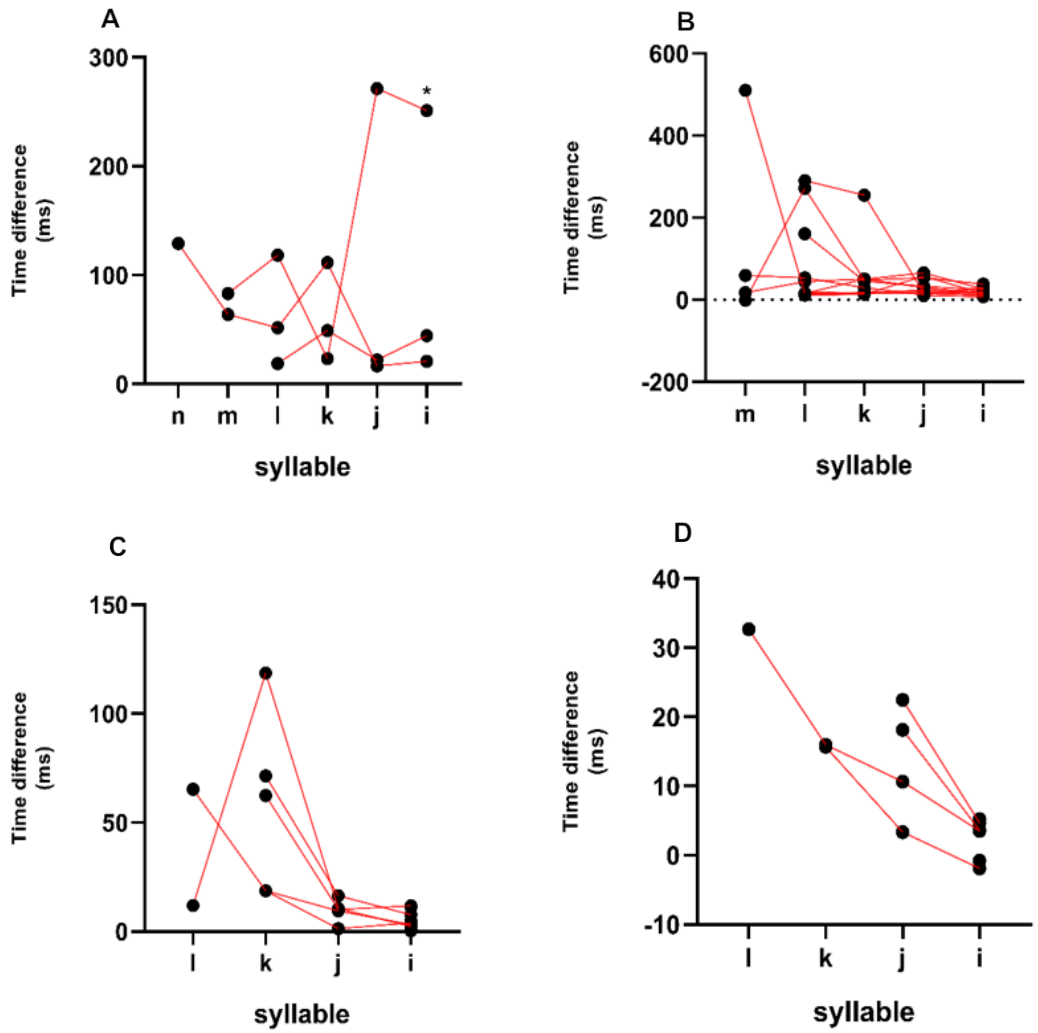

Fig.20 - Correlation offset time of different INs with that of their expirations. Values on y axis are EP offset time subtracted from IN offset time. x axis shows syllable labels, i representing last IN, j for second to last IN, etc.

A: Data from a single bird in directed condition. B: Data from the same bird in A but undirected conditions. C: Data from different bird in directed conditions. D: data from the same bird as C but in undirected conditions

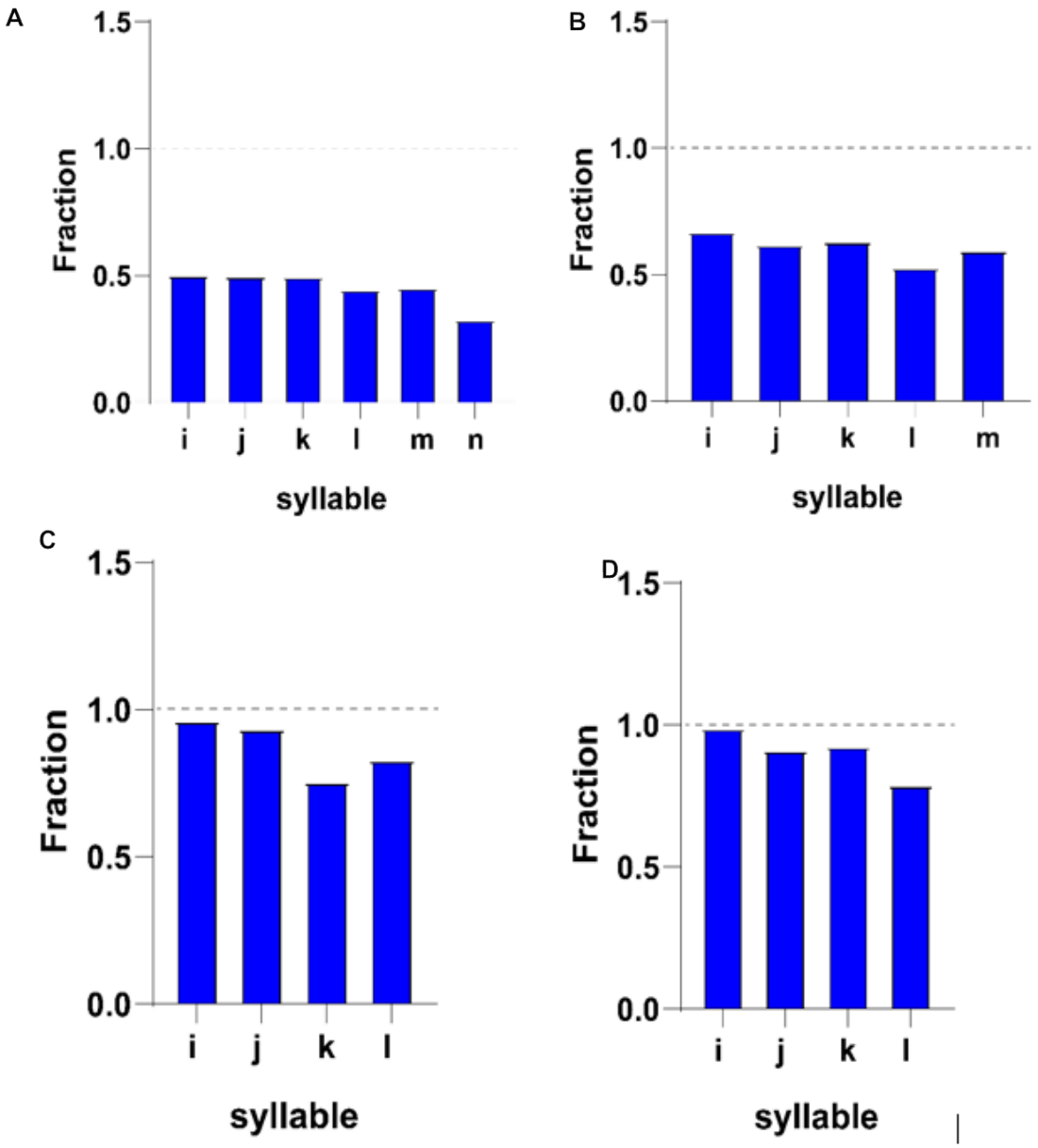

Fig.21 – Fraction of expiratory pulses occupied by vocalisations is shown for INs leading to song. A, B represents a single bird in directed and undirected conditions respectively. C, D represents data from a different bird in directed and undirected conditions respectively. x axis shows IN labels as i, j, k, and l with i being the last IN before motif.

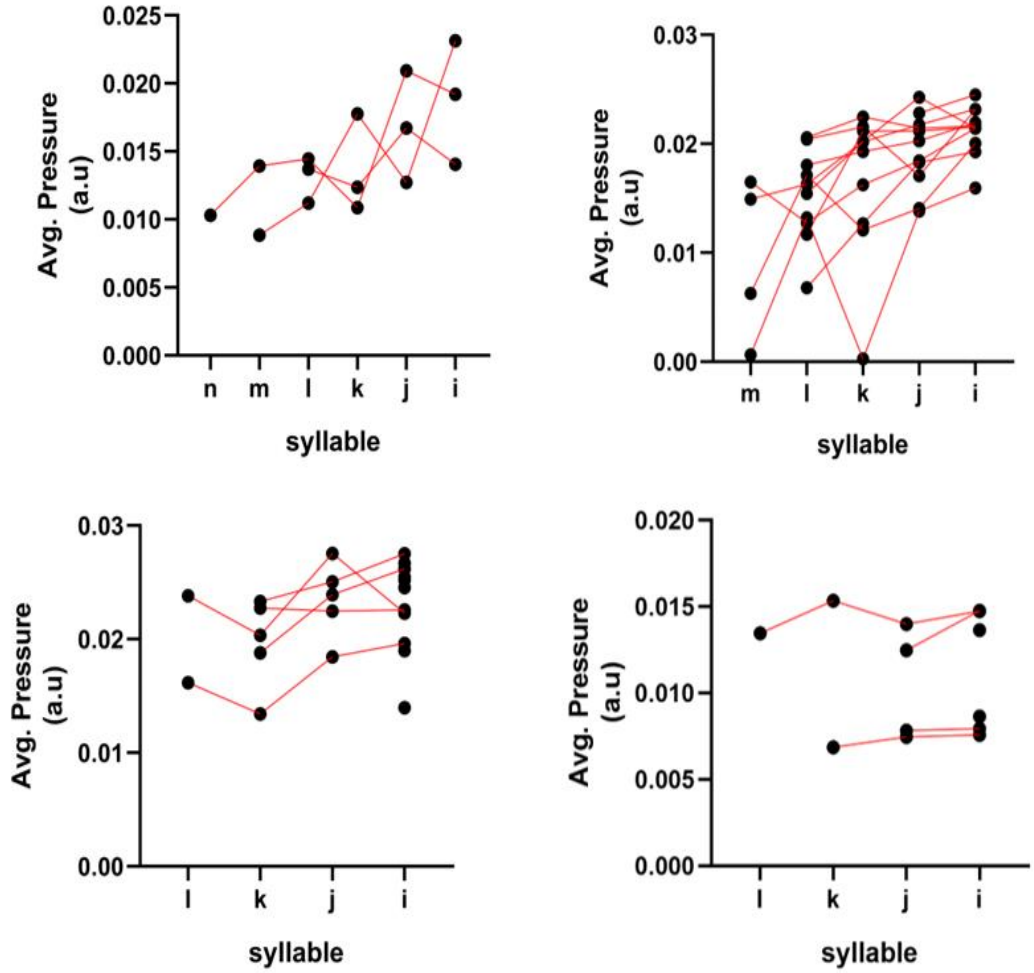

Fig.22 – Average expiratory pressure during INs leading to song is shown. A, B shows data from the same bird in directed and undirected conditions respectively. C, D represents data from a different bird in directed and undirected conditions respectively. X axis shows IN labels with i being the last IN.

Variability in expiration pattern of INs

The pattern of expiration curves for different INs was represented in a heat map (Fig.23). The color distribution of these plots would show how stereotyped are the INs at a fixed distance from song motifs. Since there were only a few bouts with a large number of INs (5-6), this pattern was looked at using expiratory data of the last 3 INs only (i, j, k).

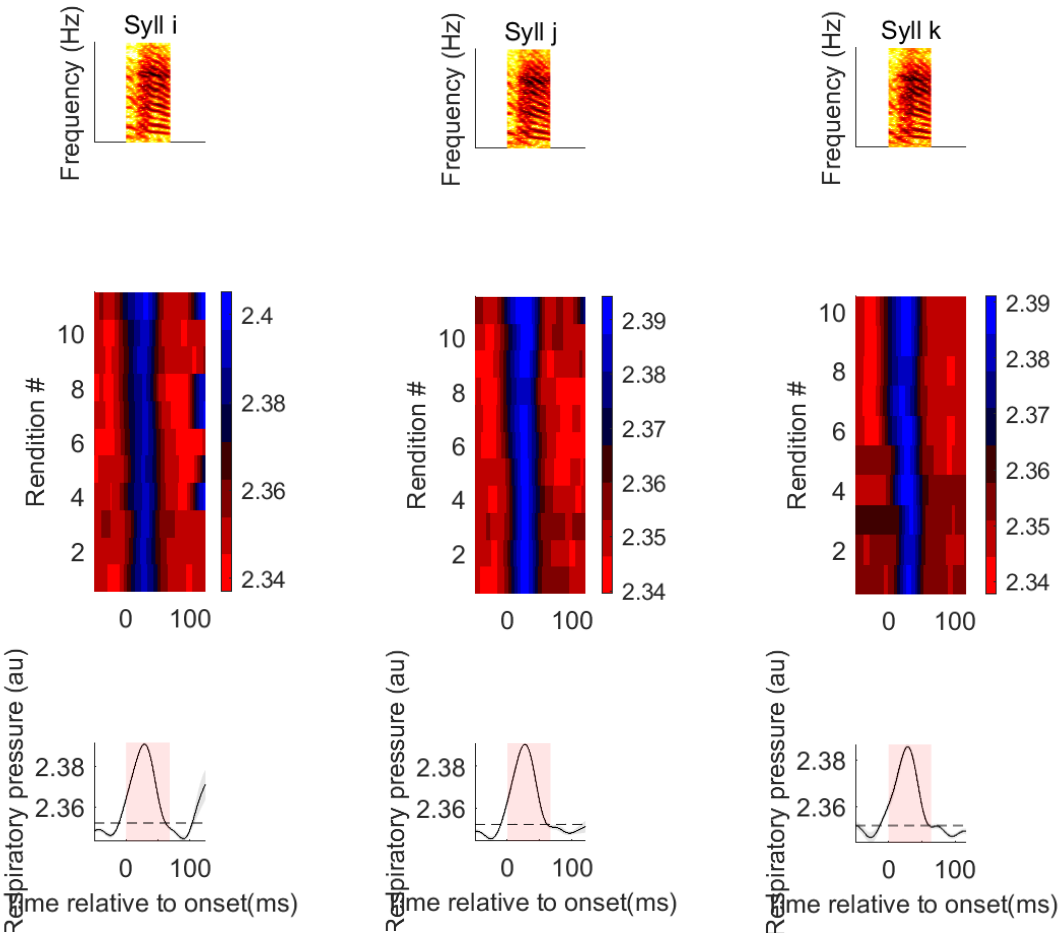

Fig.23 - Figure represents last 3 INs of the same bird with their expiratory curves (undirected).

First row shows the spectrograms of these INs i, j, k where i is the last IN, j is the second to last and so on.

Second row shows heat maps for these INs in the same order as above. On the y axis number of renditions are marked. X axis shoes time with 0 being the onset of all the INs. Pressure values assigned for colours are shown along with each plot.

Third row shows the expiratory curves of the same INs as above averaged over multiple renditions. X axis is time relative to IN onsets and y axis shows pressure values in arbitrary units. The red patches in these figures mark IN boundaries averaged over renditions. Grey patch over the expiration curves shows the variability at each point (standard error).

From Fig.23, last row, it is evident that last 3 INs have characterised expiratory curves associated with them. The small standard error (grey patch) shows this higher stereotypy of these INs.

The uniform distribution of the colours along the y axis of the heat maps (middle row) also shows the same thing. There is repeatability in the expiratory patterns of INs close to song motif. To know if this repeatability is exclusive or higher for the las INs compared to first INs, more song bouts with large number of INs are needed.

The stereotypy also includes the short inspiration (mini breath) at the end of the last IN, i in all renditions which is expected because it precedes a song syllable all the time. The summary of these patterns is shown in Fig.24 with expiratory traces of 5 INs averaged across their renditions (with very few for l and m) aligned such that the onsets of all of them coincide.

This plot also summarises the increase in expiratory pressure toward the last IN (red) and shows the occurrence of mini breath after it. The coloured patches around these curves represents the variability or standard error. Reduction in the width of these patches as we go from m to i (pink to red), could be indicating that INs become more stereotyped towards song. But data from more birds with higher number of INs in the song bouts is needed to conclude this observation.

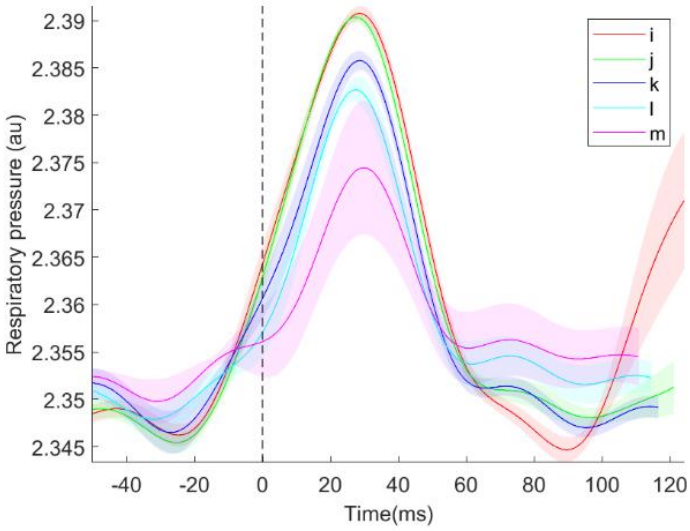

Fig.24 – Expiratory curves during different INs averaged over multiple renditions are shown (i, j, k, l, m).

X axis shows time relative to IN onset. Y axis shows respiratory pressure in arbitrary units.

Different colours represent INs with different index or distance from motif onset.

Patches with same colours around the curves represent variability or standard error at each point.

Discussion

This study aimed to assess the respiratory patterns during introductory notes (INs) of zebra finch songs, in the context of motor preparation, and comment on the role of respiration as a motor correlate of the preparatory neuronal activity. For this, respiratory patterns of zebra finch during their song were visualized by recording thoracic air sac pressure along with the song, and correlations between them were characterized.

The methods we followed included cannulation of the air sac of the birds, making a pressure sensor circuit, and making birds sing in the recording setup. Over the past months, the surgical procedure for air sac cannulation has been standardized with improvement in the survival rates of the birds. However it can be still improved, based on the knowledge of air sac anatomy we'll gain from more post-mortem surgeries of the bird. The surgical procedure can be improved also by obtaining a better CT scan of the bird, with finer visualization of soft tissues. The pressure sensor backpack has been optimized to the smallest size and weight possible, which needs no further modification as the birds are able to sing while carrying it. However, The connection from the backpack to the commutator sometimes makes birds distracted, affecting the frequency of their singing. This could be tackled with some kind of a wireless recording set-up, which we couldn't pursue this time due to the expense and standardization required. The noise control during directed song recording can be further improved by soundproofing of the female bird's cage, adding transparent separation between male and female birds. This is partially taken care of with the use of videos instead of live female birds for direct recording, although not all male birds are equally responsive to the video. Male birds also found to sing occasionally towards a dummy bird, which could be another direction to follow. Other technical challenges were noises with unidentified sources causing wrong segmenting of the respiration cycles. Any error in identifying individual inspiratory and expiratory cycles would lead to wrong values in the parameters we analyze. This could be taken forward by either coming up with measures for noise reduction or improving the script to incorporate this noise. The noise can be reduced with more accurate implantation and it would give a stronger pressure signal. The current programme we use considers the mean pressure over the entire duration of each file as the baseline of respiration. This baseline seems to

fluctuate even during small time windows, which could be because of the movement of the bird disturbing the tubes. To improve the programme, we could come up with a more reliable definition for the respiration baseline, which takes into account these fluctuations.

Respiratory patterns during the song were recorded from four birds with two of them singing in both directed and undirected contexts. These data show higher amplitude expiratory pulses (EP) associated with every vocalization. In agreement with existing literature each song syllable occurs during a single expiratory pulse with single mini-breaths filling the silent gaps between them. Just like acoustic properties, Expiratory curves of different song syllables show a unique profile.

Single expiratory pulses exist for introductory notes as well. However, the silent gaps between them can be filled by single inspiration or quiet respiration. The temporal correlation between onset and offset of vocalization and that of associated expiration did not show a significant difference across IN, song syllables, and calls. Nonetheless, the onset and offset of expiration occurs very close to those of vocalization with a tendency to wrap around them. Other properties such as average expiratory pressure and fraction of expiratory pulses covered by vocalization did not show any difference across IN, song syllables, and calls.

Looking at the individual INs, we observed consistent patterns in temporal correlation of INs and expiration, and average air sac pressure, but not in the fraction of expiratory pulses occupied by vocalization. IN onset becomes more coupled with expiration onset as they approach song motifs. Also, the time difference between IN onset and expiration onset converges to a common value. The same trend exists for IN offset and expiration offset. Average expiratory pressure consistently increases moving towards the last IN in almost every song bout. But it doesn't seem to be converging a common value across bouts. This increase in pressure could be attributed to an increase in amplitude of sound towards song (Rao et al, 2019). The increase in temporal coupling suggests an increase in fraction of expiration covered by vocalization towards the last IN, but this is not apparent from our data. We found stereotyped patterns from the expiratory curves of the last three INs averaged over rendition. The variability in these patterns seem to be decreasing as it approaches the last IN, but it is not seen in all birds. The parameters we analyzed did not show any difference between directed

and undirected conditions (2 birds). But this can't be concluded without data from more birds.

Based on these results, I think averaging properties across different INs and comparing them to different vocalizations does not give any useful perspectives on motor preparation. Looking at individual INs and grouping INs with the same distance from the motif together shows modulations respiration associated with them, which could have preparatory roles. The one-to-one relationship between number of INs and number of high amplitude EPs suggest that the pre-bout modulations in pressure amplitude occur only with INs. However, respiratory tempo can vary even before INs, which could also be part of motor preparation (Cooper et al., 2022). One of the birds we've looked at had only a single IN before motif with no higher amplitude EPs before this IN. This suggests that we might not see any distinct silent expiratory pulses before songs of birds without INs, contrary to what we predicted based on their HVC neuronal activity (Rajan, unpublished data). One could think of the reason for not having extra high amplitude EPs in the bird to be the very short motif. But it is already shown that the number of INs have no correlation with properties of song motifs, such as length of bout. We could still look for a distinct single expiratory pulse before the motif of IN-less birds as it might be helping them to reach the ready state before the initiation of song. This is unlikely and would be verified in future experiments.

We plan to expand this study to other species of songbirds. We could see how respiratory patterns are during INs and songs with less stereotypy such as the songs of the Bengalese finch. Even if we do not find any motor correlates of preparatory activity in respiration, we can try to see what is happening in the respiratory centers of the brain during song production. The motivation to look for this is the fact that the downstream neural pathways from RA have destinations in the vocal organ syrinx as well as the respiratory muscles. However, this can be challenging because these centers are located in the brain stem, making it difficult for electrode implantation. We can compare respiratory patterns with neuronal activity in RA (Rao and Rajan, unpublished data) and look for correlations.

Reference

Alonso, RG, Trevisan, MA, Amador, A, Goller, F, and Mindlin, GB (2015). A circular model for song motor control inserinus canaria. Front Comput Neurosci 9.

Ames, KC, Ryu, SI, and Shenoy, K V. (2019). Simultaneous motor preparation and execution in a last-moment reach correction task. Nat Commun 10.

Aronov, D, Andalman, AS, and Fee, MS A Specialized Forebrain Circuit for Vocal Babbling in the Juvenile Songbird.

Armstrong, E, and Abarbanel, HDI (2016). Model of the songbird nucleus HVC as a network of central pattern generators. J Neurophysiol 116, 2405–2419.

Athanasiadou, A, Buitelaar, JK, Brovedani, P, Chorna, O, Fulceri, F, Guzzetta, A, and Scattoni, ML (2020). Early motor signs of attention-deficit hyperactivity disorder: a systematic review. Eur Child Adolesc Psychiatry 29, 903–916.

Churchland, MM, Yu, BM, Ryu, SI, Santhanam, G, and Shenoy, K V. (2006). Neural variability in premotor cortex provides a signature of motor preparation. Journal of Neuroscience 26, 3697–3712.

Daliparthi, VK, Tachibana, RO, Cooper, BG, Hr Hahnloser, R, Kojima, S, Sober, SJ, and Roberts, TF (2019). Transitioning between preparatory and precisely sequenced neuronal activity in production of a skilled behavior.

Day, BL, Rothwell, JC, Thompson, PD, De Noordhout, AM, Nakashima, K, Shannon, K, and Marsden, CD (1989). DELAY IN THE EXECUTION OF VOLUNTARY MOVEMENT BY ELECTRICAL OR MAGNETIC BRAIN STIMULATION IN INTACT MAN EVIDENCE FOR THE STORAGE OF MOTOR PROGRAMS IN THE BRAIN.

Doupe, AJ, and Kuhl, PK (1998). BIRDSONG AND HUMAN SPEECH: Common Themes and Mechanisms.

Dunn, AM, and Zann, RA (1996). Undirected song encourages the breeding female zebra finch to remain in the nest. Ethology 102, 540–548.

Elemans, CPH, Mead, AF, Rome, LC, and Goller, F (2008). Superfast vocal muscles control song production in songbirds. PLoS One 3.

Fee, MS, Kozhevnikov, AA, and Hahnloser, RHR (2004). Neural mechanisms of vocal sequence: Generation in the songbird. In: Annals of the New York Academy of Sciences, New York Academy of Sciences, 153–170.

Franz, M, and Goller, F (2002). Respiratory units of motor production and song imitation in the zebra finch. J Neurobiol 51, 129–141.

Franz, M, and Goller, F (2003). Respiratory patterns and oxygen consumption in singing zebra finches. Journal of Experimental Biology 206, 967–978.

Ghez, C, Hening, W, and Gordon, J (1991). Organization of voluntary movement.

Glaze, CM, and Troyer, TW (2006). Temporal structure in zebra finch song: Implications for motor coding. Journal of Neuroscience 26, 991–1005.

Hartley, RS, and Suthers, RA (1989). Comparative Neural, and Physiology A. e~av, or~, Airflow and pressure during canary song: direct evidence for mini-breaths.

Jarvis, ED, Scharff, C, Grossman, MR, Ramos, JA, and Nottebohm, F (1998). For Whom The Bird Sings: Context-Dependent Gene Expression.

Kalra, S, Yawatkar, V, James, LS, Sakata, JT, and Rajan, R (2021). Introductory gestures before songbird vocal displays are shaped by learning and biological predispositions. Proceedings of the Royal Society B: Biological Sciences 288.

Klein-Flügge, MC, Nobbs, D, Pitcher, JB, and Bestmann, S (2013). Variability of human corticospinal excitability tracks the state of action preparation. Journal of Neuroscience 33, 5564–5572.

Konczak, J, and Abbruzzese, G (2013). Focal dystonia in musicians: Linking motor symptoms to somatosensory dysfunction. Front Hum Neurosci.

Lara, AH, Elsayed, GF, Zimnik, AJ, Cunningham, JP, and Churchland, MM (2018). Conservation of preparatory neural events in monkey motor cortex regardless of how movement is initiated.

Long, MA, and Fee, MS (2008). Using temperature to analyse temporal dynamics in the songbird motor pathway. Nature 456, 189–194.

Maina, JN (2002). Structure, function and evolution of the gas exchangers: comparative perspectives.

Marinelli, L, Quartarone, A, Hallett, M, Frazzitta, G, and Ghilardi, MF (2017). The many facets of motor learning and their relevance for Parkinson's disease. Clinical Neurophysiology 128, 1127–1141.

Mclelland, J (1989). Anatomy of the Avian Cecum.

Méndez, JM, Dukes, J, and Cooper, BG Preparing to sing-Respiratory patterns underlying motor readiness for song 1 2.

Morris, D THE REPRODUCTIVE BEHAVIOUR OF THE ZEBRA FINCH (POEPHILA GUTTATA), WITH SPECIAL REFERENCE TO PSEUDOFEMALE BEHAVIOUR AND DISPLACEMENT ACTIVITIES by.

Nottebohm, F, Stokes, TM, and Leonard, CM Central Control of Song in the Canary, Serinus canarius.

Piiper, J, and Scheid, P (1999). Modeling oxygen availability to exercising muscle.

Rajan, R (2018). Pre-bout neural activity changes in premotor nucleus HVC correlate with successful initiation of learned song sequence. Journal of Neuroscience 38, 5925–5938.

Rajan, R, and Doupe, AJ (2013). Behavioral and neural signatures of readiness to initiate a learned motor sequence. Current Biology 23, 87–93.

Rao, D, Kojima, S, and Rajan, R (2019). Sensory feedback independent pre-song vocalizations correlate with time to song initiation. Journal of Experimental Biology 222.

Rodríguez-Saltos, CA, Bhise, A, Karur, P, Khan, RN, Lee, S, Ramsay, G, and Maney, DL (2023). Song preferences predict the quality of vocal learning in zebra finches. Sci Rep 13.

Schmidt, MF, and Goller, F (2016). Breathtaking songs: Coordinating the neural circuits for breathing and singing. Physiology 31, 442–451.

Schmidt, MF, and Martin Wild, J (2014). The respiratory-vocal system of songbirds: Anatomy, physiology, and neural control. In: Progress in Brain Research, Elsevier B.V., 297–335.

Shenoy, K V., Kaufman, MT, Sahani, M, and Churchland, MM (2011). A dynamical systems view of motor preparation. Implications for neural prosthetic system design. In: Progress in Brain Research, Elsevier B.V., 33–58.

Svoboda, K, and Li, N (2018). Neural mechanisms of movement planning: motor cortex and beyond. Curr Opin Neurobiol 49, 33–41.

Terband, H, Maassen, B, Guenther, FH, and Brumberg, J (2009). Computational neural modeling of speech motor control in childhood apraxia of speech (CAS). Journal of Speech, Language, and Hearing Research 52, 1595–1609.

Veit, L, Aronov, D, and Fee, MS (2011). Learning to breathe and sing: development of respiratory-vocal coordination in young songbirds. J Neu-Rophysiol 106, 1747–1765.

Wild, JM, Goller, F, and Suthers, RA (1998). Inspiratory muscle activity during bird song. J Neurobiol 36, 441–453.

Zhang, Y, Zhou, L, Zuo, J, Wang, S, and Meng, W (2023). Analogies of human speech and bird song: From vocal learning behavior to its neural basis. Front Psychol 14.

Supplementary Information

A. Detailed protocol for air-sac cannulation
B. Surgery – Bird statistics
C. Datasheets (pressure sensor, Amplifier)
D. PCB designs (Eagle Autodesk files, Gerber files)
E. Matlab Scripts
F. Surgery/Post-mortem Videos