

WHEN DOES SONG BEGIN?
EXPLORING PREPARATORY FEATURES
OF SONG RESPIRATION

by

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ABSTRACT

Human language is an action wherein one plans for, produces, and terminates sound production. Errors in motor planning for initiation and cessation of speech lead to vocal dysfluency. Motor control of respiration is critical for the myoelastic aerodynamic mechanism of sound generation that is used by humans and many other vocalizing animals. Developing our understanding of how the forebrain directly modulates brainstem respiratory circuitry to produce sound is essential for understanding language initiation. Songbirds are an animal model for speech production in humans because of the similarities between song learning and production and language acquisition and speech production. Zebra finches (*Taeniopygia guttata*) sing a learned song that is composed of a stereotyped sequence of song syllable (motif), which is preceded by a variable number of introductory notes (1-7 notes). Here we explore whether the respiratory patterns producing introductory notes are a form of song motor preparation by comparing similarity in respiratory patterns during the introductory note sequence compared to the first song syllable. The results indicate that introductory notes are not preparatory for song, but rather serve as a form of motor practice to refine their production. As the sequence progresses, there is a pattern of decreasing duration of introductory notes as they approach song, particularly during the expiratory pulses of the sequence, which supports the notion that introductory notes are used by birds to modulate their respiratory rate during quiet breathing to generate the faster respiratory tempo needed during song production. Overall, the study sheds light on the role of introductory notes in zebra finch song production and provides a foundation for future studies aimed at exploring the neural circuits necessary for preparing to sing.

INTRODUCTION

All movements are thought to have been planned and readied in the brain before they are initiated (Ackermann and Ziegler, 2010). When we make a decision to perform a particular action, our brain prepares the necessary motor commands to execute that action. For example, when we decide to speak, our brain must plan and prepare the movements required for modified breathing, vocalizing, and shaping the vocal tract. Motor planning deficits, which can occur in conditions such as autism and speech apraxia, can impair the ability to prepare for and execute motor actions effectively. Therefore, understanding the relationship between decision-making and preparation for motor action is critical for advancing our knowledge of normal and disordered motor behavior.

However, the specific type of preparation that occurs before self-initiated, learned sequences of movements is not well understood. In order to improve our understanding of typical and atypical speech and language, it is necessary to examine the motor preparation of nonhuman animals. Songbirds are a useful animal model for studying the processes of language acquisition and production. Singing in songbirds is a complex learned behavior, and it shares many parallels with human speech (Doupe and Kuhl, 1999). They share similarities with humans in terms of developmental features of vocal imitation, genes for vocal learning, sound production mechanisms, and brain circuits controlling song learning and production. Although birdsong lacks some of the features found in human speech, such as the ability to convey abstract meanings and flexible associations, the similarities in the developmental and neural processes between the two make songbirds useful models for studying language acquisition in humans (Doupe and Kuhl, 1999).

Birdsong and speech share commonalities in their composition, consisting of ordered strings of sounds separated by brief, silent intervals. The most basic unit of a birdsong is a note, which appears as a continuous acoustic unit on a sound spectrogram. A syllable, on the other hand, comprises two or more notes clustered together, separated by silent intervals on either side of the syllable. These silent intervals occur during the mini-breaths the birds take to replenish and recover air supply. Syllables are grouped to form a motif, which is a phrase that is sung in a fixed order as a unit, with a variable number and structure between individual birds. Each bird's note, syllable structure, and syllable order creates a unique, individual-specific song (Doupe and Kuhl, 1999). The syntax of birdsong refers to the specific timing and ordering of these syllables within the motif, which parallels human language grammar, allowing birdsong to be used as a model for human language.

For an animal to display vocal learning, it is essential to acquire control over the muscles responsible for modulating respiratory patterns. In mammals and birds, sound is produced by converting aerodynamic energy into acoustic energy using their vocal organs: this occurs in the larynx in humans and the syrinx in birds. When expiratory muscles contract, they generate a positive air pressure head. In birds, this muscle activation causes the ribcage to compress the air sacs that are closely linked to the body wall. The generation of sound is limited by respiratory capacity, which not only affects the duration of sound production, but also determines when sound production can begin based on current respiratory conditions. Thus, studying how the nervous system prepares the respiratory system for phonation is crucial for comprehending the motor preparation involved in vocal production, particularly in relation to the evolution of pallial control over learned vocal behaviors.

In this study, preparatory behavior is defined as any changes in respiration that may aid in the generation of singing. Through analysis of various bird species and contexts, birds have been seen to speed up their respiratory tempo and increase the time spent inhaling prior to singing. Since singing involves a fast-paced rhythm and unique patterns of inspiratory pressure, these respiratory changes are likely indicative of preparation for song initiation. These findings suggest that respiratory behavior plays a crucial role in preparing for vocalization and can potentially predict when a bird will begin to sing.

There have been limited studies conducted on changes in respiration before singing. research conducted on Waterslager canaries (*Serinus canari*) noted an increase in the tempo of the respiratory cycle before the onset of song (Hartley and Suthers, 1989). However, the specific changes in the respiratory cycle leading up to the start of the song were not measured. Other studies have investigated oxygen consumption and heart rate in zebra finches (*Taeniopygia guttata*) and observed an increase in respiratory tempo before singing (Franz & Goller, 2003; Cooper & Goller, 2006). Nevertheless, it is not clear whether the increase in oxygen consumption or heart rate before the song is due to increased respiratory activity in preparation for singing or movements associated with the presentation of the conspecific during female-directed singing.

However, there is some evidence that suggests peripheral motor patterns may be preparatory in nature. Additionally, research has shown that there are changes in neural activity in the avian song system before singing, specifically in areas such as the avian basal ganglia and pallial areas RA and HVC. Studies using in vivo single-photon calcium imaging and multiunit electrophysiological recordings in zebra finches and Bengalese finches have identified a progression of preparatory activity from pallial premotor to motor areas, and then to downstream

respiratory motor systems. Cell-type-specific calcium imaging has revealed a sub-class of neuron projecting from HVC to RA that is active before song but not during song, and extracellular RA neural activity begins to change toward burst firing patterns typical of song about 0.5-2.5 seconds before song (Daliparthi et al., 2019). Finally, there is an acceleration of the respiration rhythm in the last second before song initiation.

The previous work explored preparatory respiratory patterns prior to onset of any vocalizations. An argument has been made that the production of introductory notes serves as a preparatory motor behavior for upcoming songs (Rajan and Doupe, 2013). Zebra finch introductory notes are distinct from song in several ways. First, they are highly acoustically similar to one another. Second, they are repeated one to seven times prior to the onset of the bird's motif, which is composed of 4 to 7 acoustically unique syllables. This raises the question as to whether introductory notes are a preparatory motor behavior that facilitates singing, or a part of song and therefore not preparatory for singing the bird's motif. In this study, we investigate whether the respiratory patterns observed during the introductory note sequence are a form of song motor preparation. Our study aims to determine whether the respiratory cycle duration and amplitude of introductory notes become increasingly similar to that of the first song syllable. Our hypothesis is that if introductory notes are indeed song preparatory in nature, then the birds should produce increasingly similar motor patterns to the upcoming song motor pattern. Conversely, if the motor gestures do not become similar to song syllables, then it suggests that intro notes are produced to practice and refine the introductory notes themselves. Ultimately, the goal of this research is to shed light on the neural control of motor preparation, and these findings will serve as a foundation for future studies seeking to explore the abneural circuits

involved in preparing for singing by defining when song begins and whether introductory notes are a preparatory motor action for the upcoming song.

METHODS

Subjects. The experimental subjects consisted of five adult male zebra finches (*Taeniopygia guttata*). Female birds were not studied because only male zebra finches learn their song, and this study focused on learned preparatory behaviors before and during learned song production.

Ethics Statement and Animal Husbandry. This study is conducted with approval from the Institutional Animal Care and Use Committee at Texas Christian University (#23-02), in an animal facility. Before the experiment was carried out, finches were housed in communal cages with 4-12 birds per cage, following a 14:10 light:dark cycle. The birds had access to seeds and water ad libitum, and were given vegetables mixed with vitamins every other day, along with eggs once a week.

Surgical Procedure. Each bird was habituated to holding the pressure transducer on its back for a period of 3-5 days prior to surgical implantation of the cannula. The transducer was held in place by a Velcro elastic band placed around the bird's thorax. A counter-weighted balance arm was used to offset the weight of the pressure transducer and accompanying wires to facilitate free movement within the cage. Surgical procedures were performed under isoflurane anesthesia (1-2%) and all efforts were made to minimize pain and distress. Prior to insertion of the cannula, absence of pain response was confirmed by gently pinching a talon. Subsyngal air pressure was recorded by inserting a small tubing (Silastic tubing, 0.76 mm I.D., Dow Corning, Midland, MI) into the anterior thoracic air sac, through an incision made below the last rib. One end of the cannula was held in place with sutures and a small amount of tissue adhesive, and the other end of the cannula was connected to a piezo-resistive pressure transducer (Fujikura XFHM 02PGR; Santa Clara, CA). A cocktail of 50:50 bupivacaine and lidocaine was administered

topically around the incision site as post-surgical analgesia. Birds were carefully monitored post-surgery until they could freely perch, hop, and move around the cage while carrying the transducer and all associated connecting wires.

Song Recording. During the experiments, the birds were individually housed in small cages (31.8x10.5x25.4 cm), surrounded by a sound-attenuating box (78.7 x 33 x 33 cm). All four sides of the sound-attenuating box were lined with 1-inch thick acoustic foam to minimize acoustic reflections. A microphone (Earthworks TC20, or SR20, Milford, NH) was suspended 14-cm in front or above the perch. Recorded acoustic data were amplified and high-pass filtered (300 Hz; RME audio, Haimhausen, Germany), then digitized (44.1 kHz sample rate, 16 bit resolution) using an analog-to-digital converter (National Instruments, NI USB-6251, Austin, TX) and Avisoft Recorder software (Avisoft Bioacoustics, Berlin, Germany). The process of digitizing song and air pressure involved setting a trigger threshold, which was roughly twice the maximum level of quiet respiration. Whenever the air pressure exceeded this threshold, the song and air pressure were recorded and saved as a file. The triggered recording started 5 seconds before the threshold was reached and continued for 5 seconds after the threshold was no longer exceeded. As a result, each file contained at least 10 seconds of respiratory and microphone data. This method resulted in a collection of files that contained song, calling, defecation, and periods of quiet breathing surrounding these events. Data were stored in multichannel, 16-bit WAV files with a 22.05 kHz sample rate directly onto the hard drive.

Song Identification. Song files were segmented from the collection of all of the data files using a custom-written program in LabVIEW (version 2018, Austin Texas). Figure A shows an example recording of a zebra finch song used for data analyses. Zebra finches sing a learned song that is composed of a stereotyped syllable sequence, known as motif. Each motif is made up

of a repeated sequence of syllables (3–8 syllables) that are acoustically unique from one another. A song bout is defined as one or more repetitions of the motif. Prior to singing the first motif of the song bout, birds sing a variable number of introductory notes (1-7 notes). Time = 0 is defined as the inspiration preceding the first song syllable in the motif. Each introductory note is labeled as -1 EP/IP to -N EP/IP depending on the number sung by the bird. Ambient pressure is estimated as the midpoint between peak negative and positive pressures during quiet breathing (Fig. A, yellow line). Supra-atmospheric pressure corresponds to expiration, while sub-atmospheric pressure indicates inspiration. Following existing nomenclature, song onset is defined as the first inspiratory pulse preceding the first introductory note of the sequence (cf., Mendez et al. 2022). Inspiratory pulses during introductory notes and between song syllables are called mini-breaths (Fig. 1, B and C, yellow). Mini-breaths are generated with shorter duration and more sub-atmospheric than quiet inspiration (Hartley & Suthers, 1989). Song termination is indicated by the respiratory oscillation of quiet breathing after song bout.

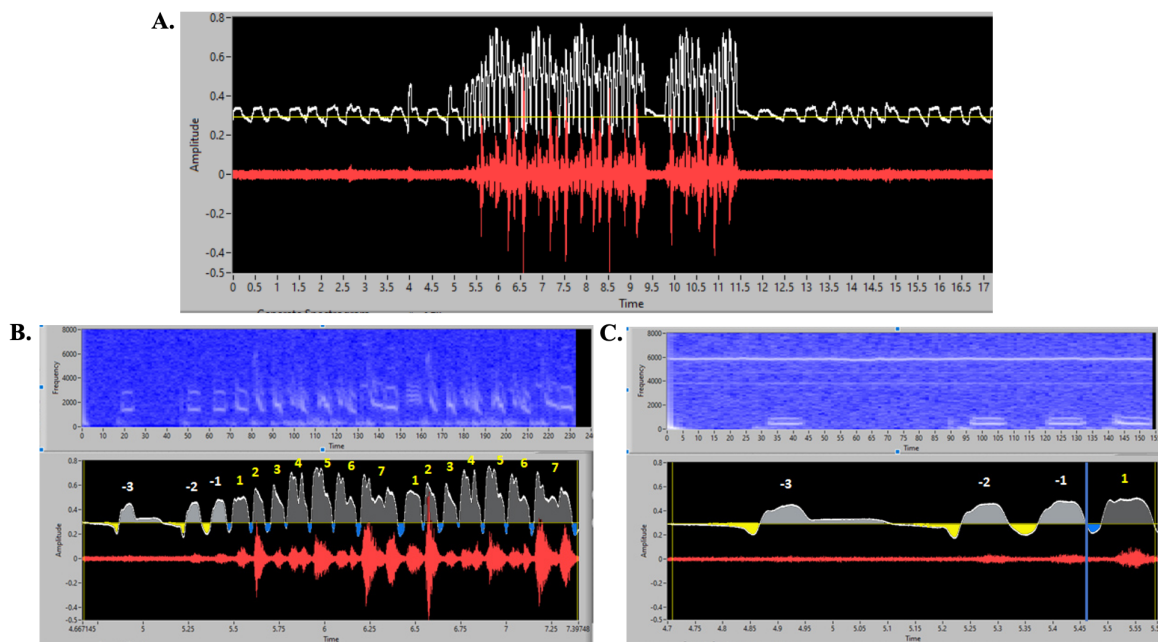


Figure 1. An example recording of a zebra finch song bout. **A)** The white trace shows the respiratory pattern of the bird before, during and after singing. The yellow horizontal line demarcates ambient air pressure; birds are

exhaling when they generate supra-atmospheric pressure and inhaling during the generation of sub-atmospheric pressurization. The red trace is the microphone recording of the bird's song. **B)** Top, a spectrogram shows the frequency (ordinate) and time (abscissa) of the sound recording from the microphone. Bottom shows an example of the initial segment of the air pressure and song from the example above. The bird produces a series of three introductory notes (-3,-2-1), followed by two repetitions of the bird's motif (7 syllables sung in a fixed sequence). The air pressure patterns were segmented based on sub- or supra-atmospheric pressure. The yellow indicates mini-breaths (sub-atmospheric pressure) during introductory notes and blue during song. The light and dark grey indicate expiratory pulses generating introductory notes and song syllables, respectively. The duration and peak pressure was quantified for the introductory notes and the first syllable of the bird's song. **C)** The respiratory patterns producing introductory notes and the first song syllable were segmented and expanded for analysis. The spectrogram (top) shows the harmonic structure of the introductory notes, and below is the air pressure. Birds generate longer duration mini-breaths (yellow) during the initial introductory notes, and gradually shorten them as the bird approaches motif onset (blue vertical line). The expiratory pulses generating introductory notes (light grey) similarly shorten in duration as bird's approach motif onset. For comparison, the first mini-breath (light blue) and song syllable (dark grey) are shown. Song syllables are characterized by variable duration expiratory pulses and mini-breaths, although mini-breaths are less variable than the expirations generating the bird's syllables.

Data Analysis

1. Descriptive Features of Introductory Note Respiratory Patterns

Within the collection of song files for each individual bird, the onset of termination of introductory notes were manually identified by trained experimenters and ambient pressure was identified. The duration and peak amplitude of the inspiratory and expiratory events during the sequence of introductory notes preceding song were quantified and saved for subsequent analyses. For the duration of the introductory note sequence, the data were then coded as -1EP, -1IP, -2EP, -2IP, -NEP, -NIP, based upon the last introductory note in the sequence being produced before song as -1EP, and the preceding mini-breath was coded as -1IP. This continued for as many introductory notes as the bird sang for that song bout. All of the birds produced at least four introductory notes, but not all of the investigated birds produced more than four introductory notes. Therefore, we limited our analysis to the -4EP/IP in the sequence. Because of the inherent nature of the variable number of introductory notes sung by the birds prior to the onset of each motif, there were far more repetitions of -1EP/IP than -4EP/IP. Cluster plots illustrate this data quantification (see Fig. 2). From these, average values for each introductory note in the sequence for duration and peak amplitude were calculated for each bird.

2. Quantifying Motor Gesture Stereotypy

To measure motor gesture stereotypy, we calculated the coefficient of variation for duration and peak amplitude for each respiratory pattern in the introductory note sequence.

$$CVDur = \text{Standard Deviation of Duration/Duration Mean}$$

$$CV_{PA} = \text{Standard Deviation of Peak Amplitude/Peak Amplitude Mean}$$

3. Euclidean Distance Between Motor Gestures

Each bird sings a song with a unique pattern of respiratory features generating the song syllables, and we assumed the same could be true for introductory notes. To normalize the data for comparisons across individuals, each datum in the distributions of the introductory note sequence for duration and peak amplitude was transformed to a z-score:

$$z = (xi - Xbar)/SD$$

Using the z-scores, the center of the distribution for each introductory note in the sequence was calculated for duration (D) and peak amplitude (PA) for each expiratory and inspiratory pulse for the introductory notes. The Euclidean distance (d) between these center points was calculated as:

$$d(D, PA) = \sqrt{(DT - D2)^2 + (PAT - PA2)^2}$$

We presumed that motor gestures that are more similar to one another would have a smaller euclidean distance by using two features of air pressure that change dramatically as birds

transition from quiet respiration to song. We defined the target motor gesture as either the last introductory note in the sequence (-1 EP/IP) or as the first song syllable in the motif (1 EP/IP). The z-scores were calculated including the duration and peak amplitude values for the first song syllable of the motif for the analysis comparing introductory notes to the song syllable. We postulated that if introductory notes are preparatory for song, they should approximate the first song syllable respiratory pattern. However, if introductory notes are only a form of practice for producing subsequent introductory notes, the motor gestures would not approximate the first song syllable.

4. Inferential Statistics

To quantify whether measured respiratory patterns changed across time, a repeated measures analysis of variance was calculated for the duration of the inspiratory and expiratory pulses for the introductory note sequence, for the change in the coefficient of variation, and for the change in euclidean distance. Our a priori hypothesis was that each of these variables would decrease as birds moved through the introductory note sequence. In all analyses, the alpha value was .05, and partial eta squared was calculated to report the effect size (SPSS v. 26). The post hoc analyses compared the last introductory note in the sequence (or the Target Song Syllable) to the preceding introductory notes, using the simple contrast in SPSS.

RESULTS

To test whether introductory notes might represent motor preparation for the upcoming song, we first analyzed how the respiratory patterns change during the introductory note sequence and how they transition to song-like and song motor gestures. We then explored whether the introductory note respiratory patterns transitioned in similarity to the last introductory note before song or the onset of song. Our goal was to determine if they are practicing for the introductory notes themselves or a form of motor preparation for singing the upcoming song.

1. Respiratory Analyses of the Zebra Finch Introductory Notes

Figure 2 shows the aggregated data of the duration and peak amplitude of each introductory note across the sequence for both inspiration (Fig. 2A) and expiration (Fig. 2B). -1 is the terminal introductory note before song onset ($t = 0$); -2 is the second to last introductory note, -3 is the third to last, and -4 is fourth from the last introductory note. Although some birds sang more than four introductory notes, not all birds do. The maximum number of introductory notes produced by all investigated birds was four, therefore this was the limit for our analysis. Patterns of increased uniformity in duration and peak amplitude were observed in both inspiration and expiration data as the bird continued to sing more introductory notes in the sequence. Relative to data from -4 introductory notes (Fig. 2A and B, yellow dots), which are largely outspread and longer in duration, -1 introductory notes (Fig. 2A and B, blue dots) exhibit a more closely clustered distribution and shorter duration overall.

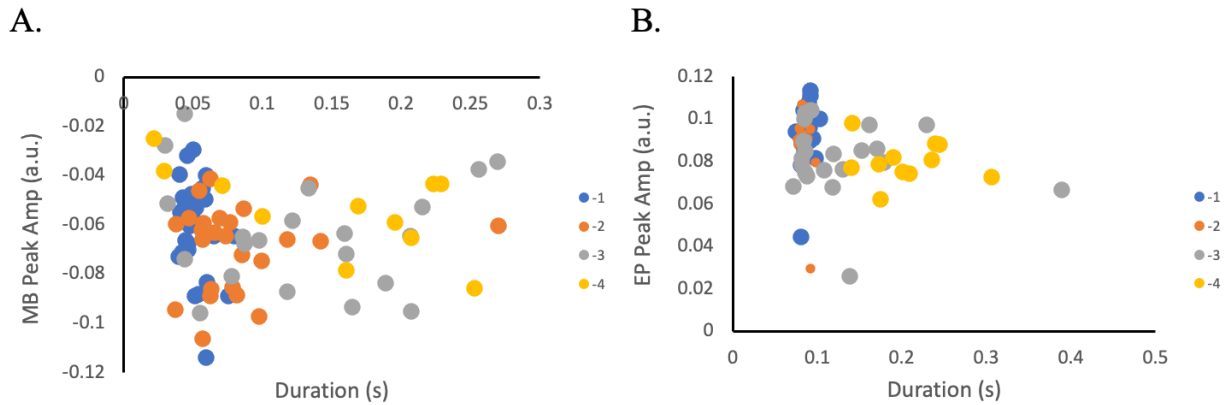


Figure 2. Example data analysis for each bird for all recorded songs. **A)** Left: Each data point corresponds to the duration and peak negative pressure (MB, inspiration). **B)** The vocal portion of the introductory note is generated by an expiratory pulse (EP, expiration) of air. The peak amplitude and duration for each measured EP is displayed.

Respiratory characteristics of duration and peak amplitude of the respiratory pulses varied across individual birds, therefore, we normalized each bird’s data using z-scores to account for individual variability using the same data set from above.

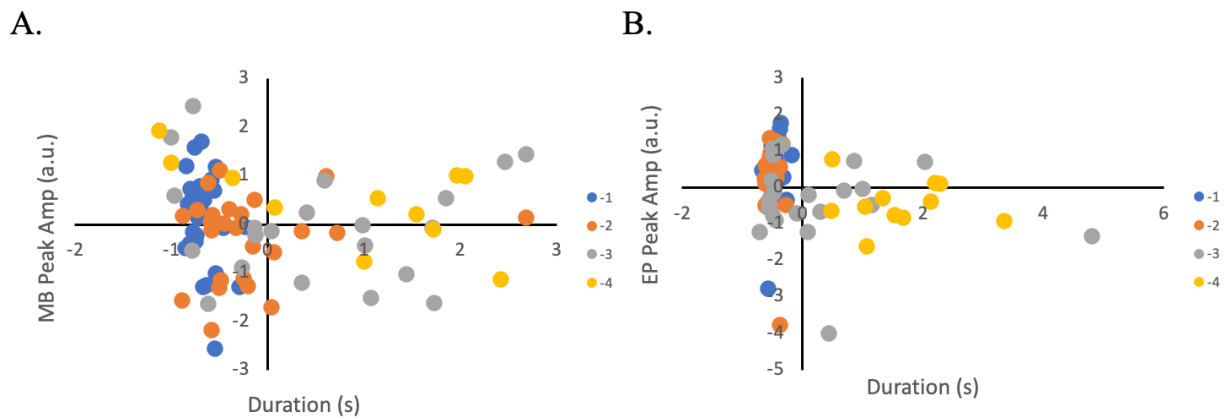


Figure 3. Data were normalized using z-scores. A,B) The data from the same bird in Figure 2 is now shown using Z-Scores for each data point of inspiratory and expiratory pulses..

Figure 3 illustrates that as birds progress through their introductory note sequence, they move toward producing shorter-duration inspirations and expirations. In this case, the birds

produce more structured, shorter duration, and higher amplitude mini-breaths. This demonstrates that birds are shifting from a less uniformed respiratory motor pattern to a more stereotyped motor gesture as they approach the last introductory note in the sequence prior to song onset.

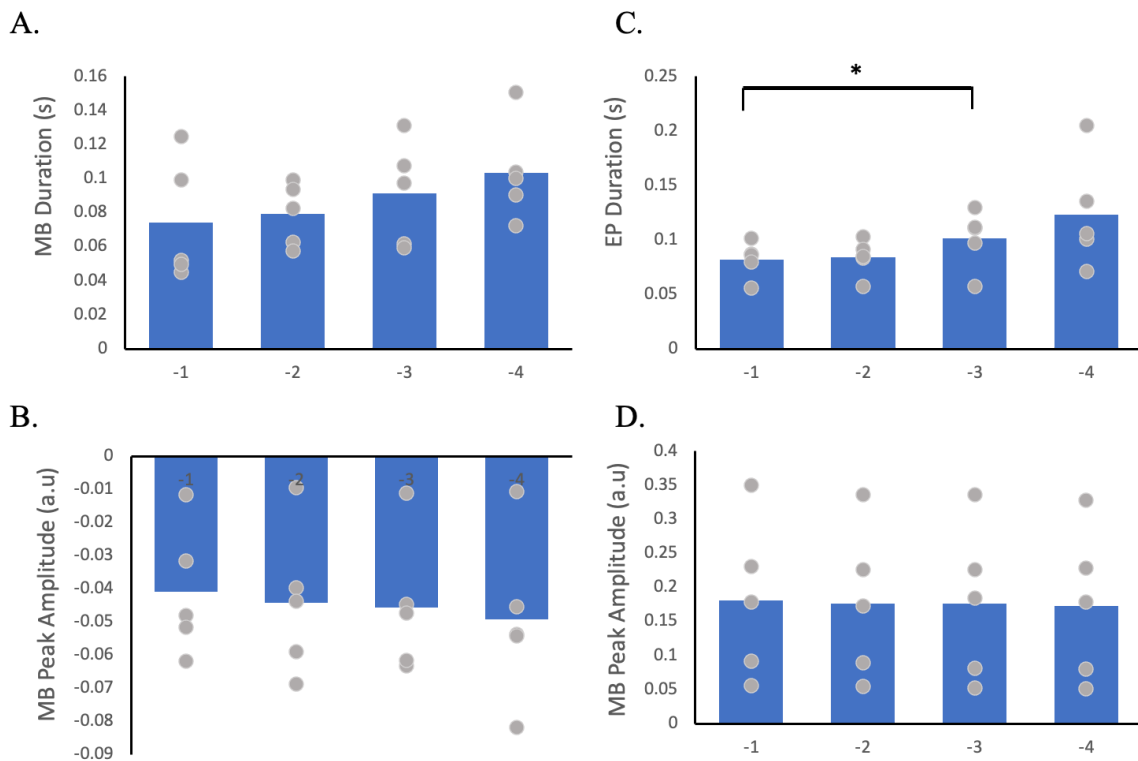


Figure 4. Group averages and individual animal changes (N = 5) in respiratory patterns during the production of introductory notes. A) The average duration for each introductory note is shown with the dots corresponding to individual animals. Three animals show a decrease in MB duration and two animals decrease and then increase in duration. C) The EP duration decreases significantly as birds repeatedly sing introductory notes. B, D) The peak amplitudes of both MB and EP do not change as a function of order within the sequence.

Next, we investigated whether the acoustic properties of introductory notes changed as each sequence progressed to the last introductory note, which we called IN -1. To characterize respiratory patterns during expiration and inspiration, we used two different features: duration and peak amplitude. The data were used to generate descriptive and inferential analysis of respiratory characteristics of the introductory note sequence. Figure 4 shows the group averages and the individual changes in respiratory pattern throughout the four introductory notes sequence. The mini-breath duration and amplitude decreased in three birds, but not others as they approached song ($F(3,12) = 1.93$, n.s.; $F(3,12) = 1.123$, n.s.). The last two introductory notes in the sequence were significantly shorter in duration than the third to last notes ($F(1,4) = 7.451$, $p = .052$, $\eta_p^2 = .651$). The expiratory pulse duration decreased as birds approached song ($F(3,12) = 3.923$, $p = .037$, $\eta_p^2 = .495$), however, the amplitude of the expiration did not change as a function of introductory note sequence ($F(3,12) = 2.722$, n.s.). Over the course of the introductory notes production, there was no significant change in peak amplitudes across both the mini-breaths and the expiratory pulses (Fig. 4B, D).

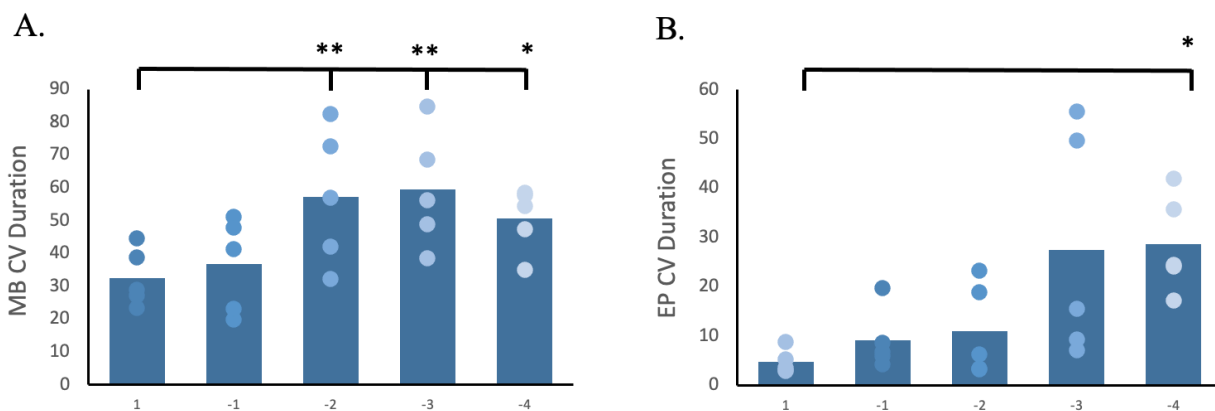


Figure 5. Motor stereotypy emerges during the production of the introductory note sequence. A) Coefficient of variation (CV) for MBs during the introductory note sequence and the first song syllable illustrates a significant increase in motor stereotypy emerging by the last introductory note MB compared to the earlier MBs in the

sequence. B) Motor stereotypy emerges early in the introductory note sequence for the EPs, as only the -4 introductory note is significantly different than the first song syllable EP.

Figure 5 addresses the stereotypy of motor control. Coefficient of Variation was used as a normalized measure of the variability of a dataset, in this case, to quantify the variability of motor stereotypy in the introductory note sequence and the first song syllable of a song. Lower values of coefficient of variation (CV) correspond to more stereotyped motor control, and higher values indicate increased motor variability. The data suggest that as the introductory note sequence progresses, there is a significant increase in motor stereotypy that becomes particularly noticeable in the last introductory note MB when compared to the earlier introductory note MBs in the sequence ($F(4,16) = 6.802, p = .002, \eta_p^2 = .630$). Motor stereotypy is evident early on in the introductory note sequence for the EPs, as only the -4 introductory note shows a significant difference compared to the first song syllable EP ($F(4,16) = 3.909, p = .02, \eta_p^2 = .497$). Prominent stereotypy observed at IN-1 suggests the last introductory note is the beginning of song onset.

2. Respiratory Analyses Compared to Target Introductory Note vs. First Song Syllable

Here, to explore the preparatory functions of introductory notes, we analyzed the properties of introductory note sequences and how they transition to song. If introductory notes serve as a form of motor practice, then preceding introductory notes in the sequence should be successively approximating IN-1, or the last introductory note in the sequence (Fig. 6, Target A). If introductory notes sequence is approaching the first syllable of motif onset, then introductory notes are preparatory in function for the preparation of song motor gesture.

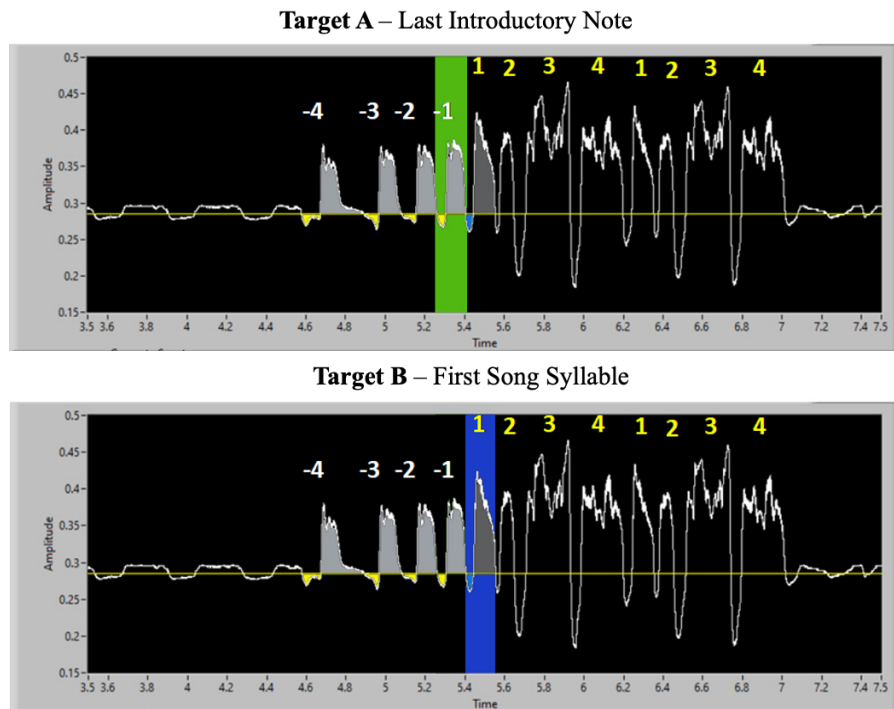


Figure 6. Illustrations of target introductory note (IN -1, Target A) and first song syllable (1, Target B)

Duration and peak amplitude data of introductory notes and first syllable were aggregated. Z-scores of the same data set were generated to account for individual variability. Figure 7 shows the Z-scores for both the MBs and EPs of introductory notes compared to either the last introductory note (Fig. 7A) or the first motif syllable (Fig 7B). The centroids, which illustrate the mean points of each distribution, were calculated and plotted in Figure 8.

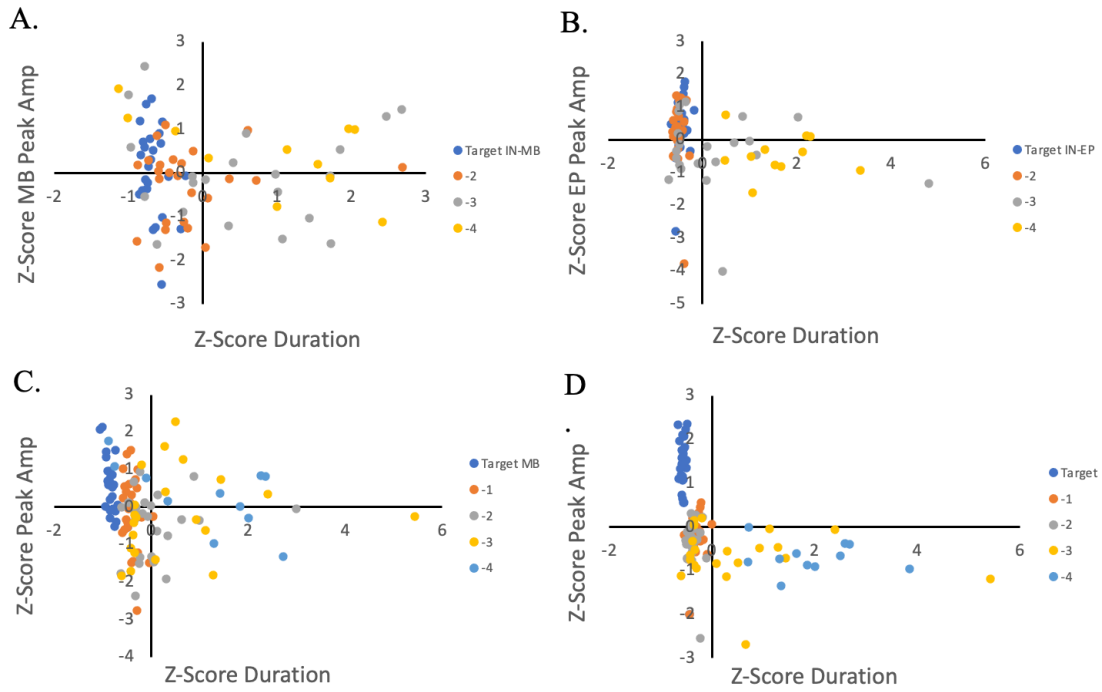


Figure 7. Normalizing data using Z-scores for both sets of targets. A, C) Z-score cluster plots of both MBs and EPs data compare to Target A: Last Introductory Note. B, D) Z-score cluster plots of both MBs and EPs data compare to Target B: First Syllable.

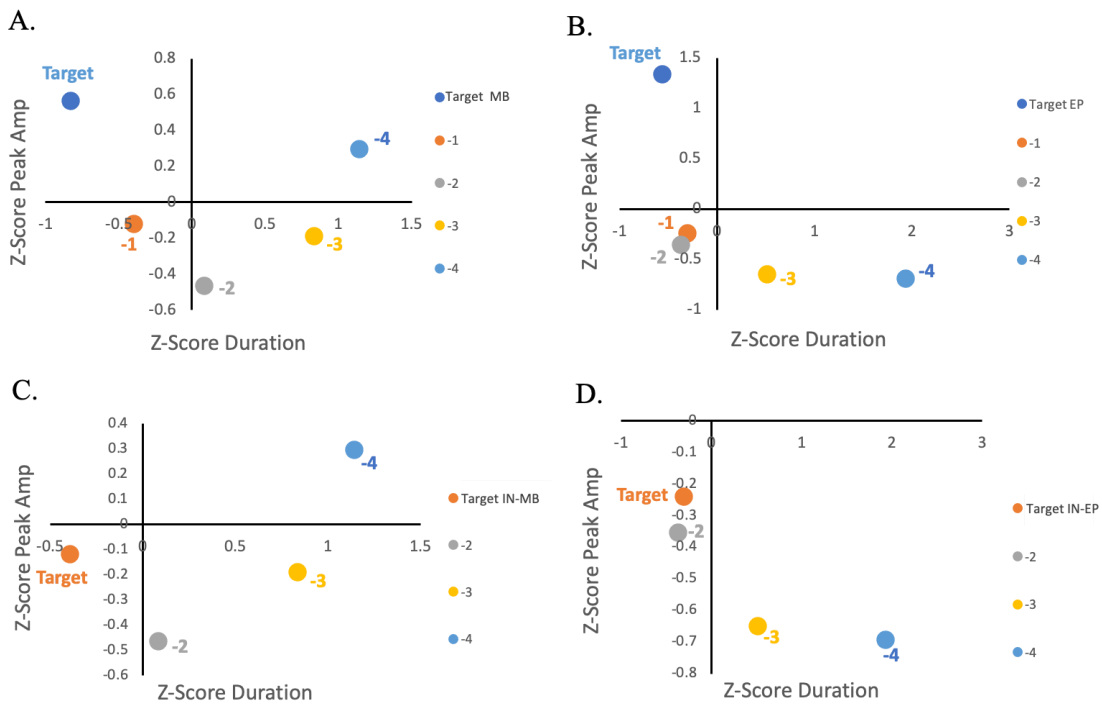


Figure 8. Centroids of the distributions from Figure 7. A, C) Centroid graphs of MBs and EPs data compare to Target A: Last Introductory Note. B, D) Centroid graphs of MBs and EPs data compare to Target B: First Syllable.

Distances between centroids and the respective target centroids were calculated as the Euclidean distance between the points. The Euclidean distance (ED) measure encompasses two variables, the distance in duration and peak amplitude of the inspiratory or expiratory phase of the respiratory cycle. The ED between centroids is a measure of similarity of the motor gesture; a motor gesture that is a close approximation to the target motor gesture is indicated by a smaller ED and conversely, more divergent motor gestures have a larger ED.

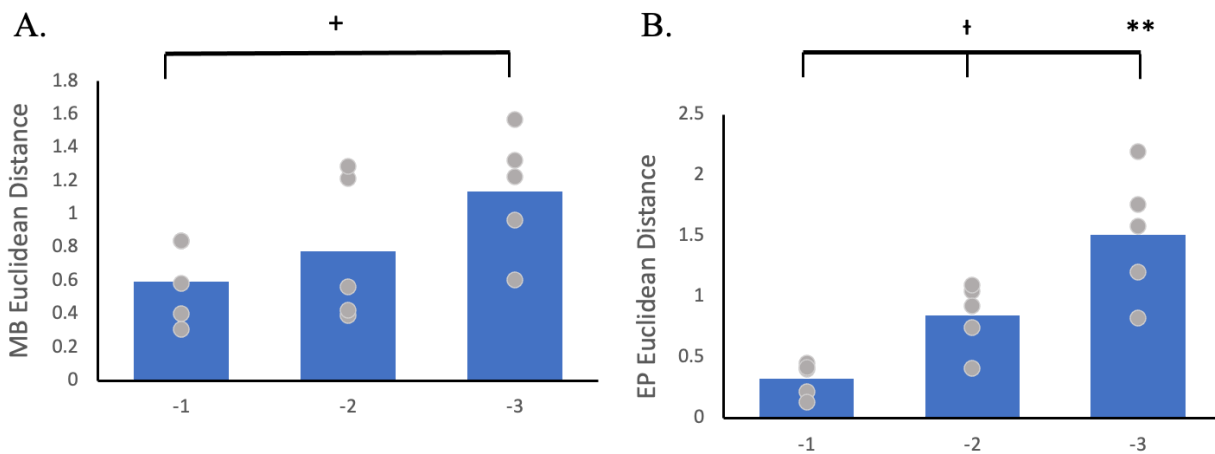


Figure 9. Changes in Euclidean distance approaching target last introductory note. A, B) The Euclidean distance for MBs and EPS decreased significantly and approached the target of the last Introductory note. † indicates $p = .014$; ** indicates $p = .002$ and + is $p < .067$

The target in Figure 9 is the last introductory note in the sequence. Here, we find the predicted preparatory pattern for both mini-breaths (Fig. 9, A) and expiratory pulses (Fig. 9, B) producing the introductory note respiratory cycle. The ED shows that as birds are producing mini-breaths, the respiratory characteristics of duration and peak amplitude begin to more closely match the terminal introductory note ($F(2,8) = 4.81, p = 0.047, \eta_p^2 = .546$). The last introductory note in the sequence is significantly further away than the third introductory note in the sequence. ($F(1,40) = 6.27, p = .066, \eta_p^2 = .611$). Similarly, as birds sing repeated introductory

notes, the respiratory characteristics of the expiratory pulses generating the vocal portion of the introductory note cycle more closely match the respiratory cycle of the last introductory note ($F(2,8) = 13.997, p = .002, \eta_p^2 = .778$). The last introductory note in the sequence is significantly further in distance from both the second to last ($F(1,4) = 13.48, p = .021, \eta_p^2 = .771$) and the third from the last ($F(1,4) = 17.14, p = .014, \eta_p^2 = .865$).

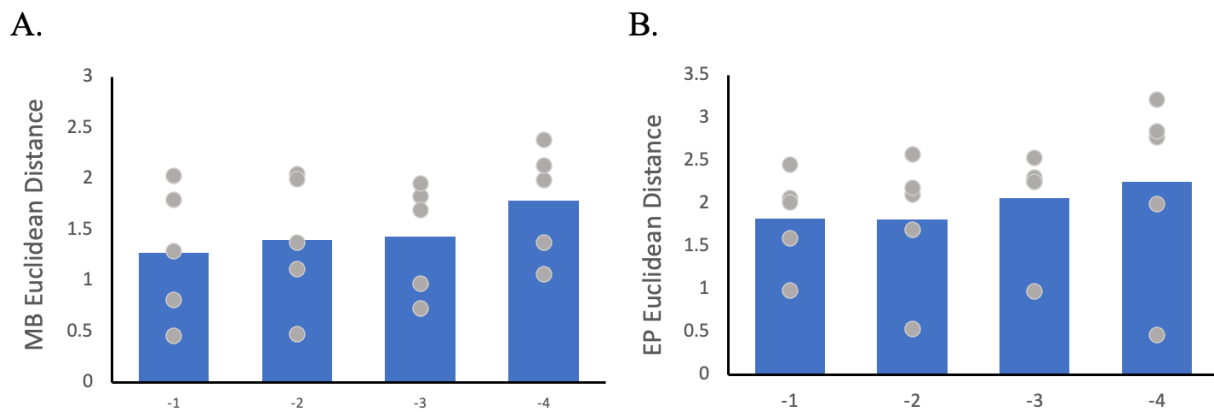


Figure 10. Changes in Euclidean distance approaching first syllable of song motif. A, B) The Euclidean distance for MBs and EPs did not change significantly compared to the target of the first song syllable.

The target in Figure 10 is the first syllable of song motif. Although there is a general reduction in the mean Euclidean distance as birds produce their sequence of introductory notes, this is not statistically significant when the target variable is the first mini-breath preceding song ($F(3,12) = 1.369, n.s.$) (Fig. 10A). Similarly, there is no evidence that birds are producing introductory notes that share similar expiratory respiratory features as the first song syllable ($F(3,12) = 1.273, n.s.$; Fig 10B).

DISCUSSION

The findings of this study provide important insights into the role of introductory notes in zebra finch song production. The results indicate that as the song progresses toward the end of the sequence, there is a pattern of decrease in the duration of introductory notes, but not in their amplitude. Significant shortening of duration is observed particularly during the expiratory pulses (EPs) of the introductory note sequence, which supports the notion that introductory notes are used by birds to modulate their otherwise slower respiratory rate during quiet breathing in order to generate a faster respiratory tempo needed during song production.

While the overall pattern of decreased duration is also seen in the inspiratory pulses, or mini-breaths (MB), of the introductory notes sequence, only three out of the five subjects exhibit this pattern mentioned above. Two individuals produced a sequence of MBs with an increased duration at the last introductory note. Zebra finches sing highly individualized songs, which may offer an explanation for the variations observed in the way the introductory notes are produced. Furthermore, deviations seen in MB pattern may be due to differences in the first syllable of song motif. If the first syllable is longer in duration and substantially higher in pressure amplitude than the introductory notes, then the preceding MB may need to be longer in duration as well to generate sufficient air supply for the production of that syllable. Future comparison of the last MBs and subsequent first syllables may prove useful in further understanding this pattern.

Additionally, the introductory note sequence analysis indicates an interesting distinction between MBs and EPs, with changes in duration and amplitude occurring independently from one another. Song is defined by this stereotyped pattern, and the emergence of stereotypy is used in this study to indicate the point of song onset. Coefficient of variation for MBs during the introductory note sequence and the first song syllable illustrates a significant increase in motor

stereotypy emerging by the last introductory note MB compared to the earlier MBs in the sequence, which indicates that song onset is likely to occur at the last introductory note. On the other hand, motor stereotypy emerges early in the introductory note sequence for the EPs, as only the -4 introductory note is significantly different than the first song syllable EP. This is reminiscent of changes in song motor patterns induced by the presence or absence of a female bird. When a female is presented, the expiratory rhythm is accelerated and more stereotyped than when the female is absent. In contrast, the presence or absence of the female has no effect on the timing or stereotypy of the inspiratory duration. This suggests that inspiration is more reflexive and less temporally modifiable, whereas expirations show a higher degree of temporal malleability, perhaps to facilitate song motor practice because sound is produced on expiration (Goller & Cooper, 2006). The change in the expiratory respiratory rhythm is established and practiced most in the sequence of introductory notes compared to the inspiratory pattern. Therefore a separation of these expiratory and inspiratory oscillatory networks may be related to vocal motor practice necessary for refining sound production during the introductory note sequence.

Following these results, our study is also interested in exploring whether these changes across introductory notes sequences were song preparatory motor behaviors. There are at least two potential hypotheses as to why birds produce introductory notes. First, it is possible that introductory notes are preparatory for the production of song, and therefore the birds should produce increasingly similar introductory motor gestures to the song motor pattern. This postulate is based on a previous study that investigated the preparation process precedes self-initiated, naturally-learned sequences of movements (Rajan and Doupe, 2013). If this did happen, then the introductory notes sequence should exhibit increasing similarity to the first

syllable of song motif. On the other hand, if introductory notes are more closely approaching the last note in the sequence, yet remain distinct from the motor gestures of song motif, then introductory notes are more likely to serve as a form of introductory note motor practice.

The results illustrate that the respiratory pattern producing introductory notes show the closest approximation to the last introductory note in the sequence and not to the first song syllable; this finding was even true for the MBs, where birds are preparing the air supply for the upcoming song syllable and are aphonous. This suggests that introductory notes may function as a form of introductory note motor practice – they simply allow the bird to refine the production of the introductory notes themselves but are not refining motor gestures of the upcoming song. In sum, the extent of introductory notes' song preparatory characteristic is only as much as being a form of motor practice to produce the next introductory note in the sequence.

A third hypothesis is that introductory notes may allow for a biomechanical enhancement of song production by bringing the thoracic cavity into a rapid movement pattern that generates the fast respiratory tempo of song. Brown-headed cowbirds (*Molothrus ater*) generate air pressure with less expiratory muscle effort when they incorporate wing movements compared to when they do not visually display during song (Cooper & Goller, 2004). The utilization of accelerating the respiratory tempo during the production of may be a similar form of increasing the biomechanical efficiency of thoracic movements to allow for a rapid, stereotyped song. The generation of introductory notes induces increasingly faster thoracic movements, which serves to overcome the inertia of a rigid and slowly moving thorax characteristic of silent respiration, and gradually transitions it to a rapidly moving thoracic cavity. This transition time may allow the zebra finches to sing a highly stereotyped song at a pace that is two to four times that of quiet breathing. If this is true, then birds that do not utilize introductory notes in their songs may

produce longer duration first song syllables, or may produce song respiratory patterns that are not orders of magnitude faster than quiet respiration. A broader investigation of species respiratory characteristics and presence and absence of introductory notes would address this question.

One crucial aspect to consider in vocal production is volitional respiratory neural control for sound production. Reflexive respiratory circuits must be actively modulated to change the ongoing timing and amplitude patterns to support the motor gestures necessary for song production. Previous studies have investigated the neural and motor correlates of vocal production in songbirds, highlighting the significance of examining neural control alongside respiratory data to better understand the mechanisms underlying vocal production. Therefore, investigating the neural changes that correspond to motor gestures can provide valuable insights into the neural mechanisms underlying vocal production. One previous study focused on investigating how the neural activity in the vocal control center of the zebra finch brain, called HVC as the proper name, relates to the motor gestures involved in singing (Daliparthi et al., 2019). Neural activity was recorded while birds performed a variety of song behaviors, including singing their full song, singing specific syllables, and producing non-song vocalizations. The results showed that the activity of individual HVC neurons was highly correlated with specific aspects of the song, such as the timing and duration of particular syllables. This suggests that the HVC is involved in the precise control of motor gestures during vocal production. Furthermore, the researchers found that the neural activity in the HVC was strongly modulated by auditory feedback. When the birds heard their own songs played back to them with specific alterations, such as changes in pitch or timing, the neural activity in HVC changed in response. This suggests

that the brain uses auditory feedback to continuously adjust and refine motor commands during vocal production.

Another relevant study examined both behavioral and neural signatures of introductory notes in relation to the initiation of song, a learned motor gesture (Rajan and Doupe, 2013), as mentioned earlier. Upon HVC-RA analysis, it was discovered that interneurons were active during introductory note sequences with a significantly higher mean firing rate than the baseline before IN. In addition, similarity between activity patterns of all pairs of last introductory notes were calculated and a strong correlation of neuronal activity was found between each pair. These results suggest that neural activity patterns were stereotyped at each introductory note position in the sequence, and that introductory notes production may be a mechanism for achieving the coordination needed to produce song vocalization, by repeatedly activating the HVC and respiratory centers across the hemispheres. Based on these findings, further investigation into the neural correlates of motor gestures during vocal production could provide insight into the mechanisms of motor control and learning in the brain. By studying the relationship between neural activity and motor gestures in more detail, it may be possible to develop a better understanding of how the brain produces and learns complex vocal behaviors.

There are several limitations to this experiment, including expanding the measures of song respiration as well as increasing the sample size. In this study, we only explored two features of respiratory patterns: duration and peak amplitude. It is possible that by studying the temporal modulation and slope of the rising and falling pressure patterns produced during each breath would provide a better understanding of the preparatory nature of the respiratory introductory note sequence. The study had low statistical power with only five birds; therefore increasing the sample size could improve the stability of the means for each unit of measure.

Additionally, the unique syntax of zebra finch song, where each bird sings a highly individualized song with differing syllables, also posed a challenge to assessing each bird on the same level, especially with a small sample size. While the measures of song duration, amplitude, and stereotypy may be well-suited for analyzing birdsong, they may need to be adjusted to better capture the characteristics of introductory notes.

Our next experiment will attempt to disrupt song onset during introductory notes. If the introductory notes are a form of motor preparation for the stereotyped motor control of song, then one should be able to disrupt song onset by perturbing either behaviorally or physiologically motor control of song. The emergence of stereotypy at the final two introductory notes implies that the birdsong has already commenced at this point in time of the introductory note sequence. This suggests that once the bird reaches this point in its song, the stereotypy has already been established and song cannot be disrupted. Future disruption studies could focus on targeting the introductory notes that come before the last two, since these are earlier in the sequence and may be more susceptible to disruption. By targeting these earlier notes, researchers may be able to gain a better understanding of the neural mechanisms involved in song production and the role of stereotypy in the process.

It is imperative to continue researching zebra finches, as well as other songbirds, to better explore animal models that closely imitate language acquisition and production in humans. Studying the preparatory activities in songbirds can provide valuable insights into motor preparation of human speech disorders like Parkinson's disease and speech apraxia, and better understand the neural mechanisms involved in speech production and how these mechanisms are affected by various disorders. The importance of communication in our daily lives cannot be overstated. It is essential for our survival and the maintenance of interpersonal relationships.

Therefore, gaining a further understanding of the animal model for this complex behavior could not only benefit those who have lost this ability but also the general population who rely on communication every day.

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