# Variability in the male zebra finch song and its role in female mate choice

A Thesis

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by

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# Certificate

This is to certify that this dissertation entitled **Variability in the male zebra finch song and its role in female mate choice** 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 Samyuktha Ramadurai** at Indian Institute of Science Education and Research under the supervision of **Dr Raghav Rajan**, Assistant Professor, Department of Biology, during the academic year 2017-2018.

Dr Raghav Rajan

Assistant Professor

Dept of Biology

Committee:

Dr Raghav Rajan

Dr Deepak Barua

This thesis is dedicated to my parents.

# Declaration

I hereby declare that the matter embodied in the report entitled **Variability in the male zebra finch song and its role in female mate choice** 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.

Samyuktha Ramadurai

9 April 2023

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# Abstract

The song of the adult male zebra finch is an example of a learned motor sequence consisting of a sequence of sounds interleaved by silent gaps. As with any motor action, the song of the zebra finch is subject to motor variability. It is interesting to study the variability in the acoustic features of syllables across different renditions of song, since previous studies have shown that zebra finches can perceive this variability. It is hypothesized that consistent songs are an honest signal of male quality, and many studies on other species of songbirds have shown that females prefer males which sing consistently, produce consistent trills, etc. My thesis aims to find out if female zebra finches can perceive variability in song, and prefer male songs which are consistent over male songs which are more variable. Two paradigms were used to test my hypothesis: A preference assay and an operant conditioning classification task experiment. It was found that females do not exhibit a preference for consistent or variable songs, despite having used multiple methods to quantify the preference of the female birds. The setup for the operant conditioning experiment was constructed and works for training the birds, but there are no results from that experiment.

# Acknowledgments

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# Contributions

| Contributor name | Contributor role |
|---|---|
| Samyuktha Ramadurai, Raghav Rajan | Conceptualization Ideas |
| Samyuktha Ramadurai, Abir Dutta, Devika Kizhakoot | Methodology |
| Raghav Rajan, Shikha Kalra, Samyuktha Ramadurai | Software |
| Samyuktha Ramadurai, Abir Dutta, Devika Kizhakoot | Validation |
| Samyuktha Ramadurai, Abir Dutta, Devika Kizhakoot | Formal analysis |
| Samyuktha Ramadurai, Abir Dutta, Devika Kizhakoot | Investigation |
| Raghav Rajan, Shikha Kalra | Resources |
| Samyuktha Ramadurai, Abir Dutta, Devika Kizhakoot | Data Curation |
| Samyuktha Ramadurai | Writing - original draft preparation |
| Raghav Rajan, Samyuktha Ramadurai | Writing - review and editing |
| Samyuktha Ramadurai, Devika Kizhakoot | Visualization |
| Raghav Rajan | Supervision |
| Raghav Rajan, Samyuktha Ramadurai | Project administration |
| Raghav Rajan | Funding acquisition |

# Introduction

People perform actions a little differently each time, however well-practised they are. For example, if a good basketball player were given many trials to perform free throws, they would manage to throw the ball in the basket in most of the trials, but would miss the basket in a few of them. This is due to the inherent motor variability that comes with any muscular action we perform. Birds exhibit similar variability in their vocalizations (Sossinka and Böhner, 1980; Ölveczky *et al.*, 2005; Cooper and Goller, 2006; Kao and Brainard, 2006; Teramitsu and White, 2006). Song birds attract mates using songs, and the males are subjected to sexual selection on the basis of the songs they sing. Until recently, the role of consistent singing in mate choice and sexual selection has been poorly understood. In the context of birdsong, song variability is interesting to explore as the song is a signal which reveals information about the signaller. The receiver of the signal, which is typically the female, can utilize the signal to get information about the male. The extent of variability which can be perceived by the receiver, and thus be relevant in communication, depends on the sensitivity of the receiver to the variations. Hence, the communicative potential of variability in different renditions of the song is worth exploring.

The Zebra finch (Taeniopygia guttata) is a good model to study song variability. This is a songbird (order Passeriformes) belonging to the family Estrildidae, and is native to central Australia. Juvenile zebra finches learn song from a male tutor, which is typically their father. A song consists of a few song motifs and the introductory notes. A song motif is a stereotyped sequence of syllables. Vocalizations of juveniles are initially highly variable, and they become more structured with age and practice (Zann, 1996; Price, 1979) The song becomes stereotyped (‘crystallized’) when the birds attain about 90 days of age. However, song learning continues throughout the lifespan of the bird, as the song becomes more and more consistent (less variable) with age (Kao and Brainard, 2006). Despite the increasing consistency, there is significant syllable-level variability in the adult zebra finch song.

It has been shown that adult zebra finches which are 4 years and older have a 40% greater consistency in their song syllable structure than adult males which are less

than 6 months old (Kao and Brainard, 2006). This syllable-level variability is an example of motor variability. It is hypothesized that rather than being a product of biological noise, motor variability could have important functions in allowing adjustment to changing environmental conditions, ageing, and injury. In the context of song learning in the zebra finch, juveniles exhibit considerable motor variability in order to match the tutor's song effectively (Ölveczky *et al.*, 2008). There is evidence that song variability is actively driven by the brain even after the bird matures, suggesting that is has a function after song crystallization (Kao *et al*., 2005; Ölveczky *et al.*, 2005; Stepanek and Doupe, 2010). It has been shown that song variation helps in adaptive modification of song features, and thus helps the bird in continuously learning and adjusting to produce a "perfect" song (Tumer and Brainard, 2007). In their study, Tumer and Brainard provided real time auditory disruption to a subset of natural variations in the pitch of targeted syllables while the birds were singing, and observed that the birds shift the mean pitch of those syllables so that they avoid incurring the auditory disruption (Tumer and Brainard, 2007). Thus, natural variations in the pitch of syllables allowed the birds to limit the pitch of the targeted syllables to a specific range when aversive feedback was provided.

Vocal consistency can differ across different social contexts. Vocal consistency is a measure of how accurately the bird repeats the acoustic features of song across different renditions of song. Consistent vocalizations depend on the generation of consistent motor commands from the brain and accurate functioning of the syringeal and respiratory muscles (Zollinger *et al.*, 2008). When adult male zebra finches sing, they produce directed songs while singing to females, and undirected songs while singing in isolation (Sossinka and Böhner, 1980). Directed songs are very consistent across renditions, while undirected songs are more variable from one rendition to another (Sossinka and Böhner, 1980; Ölveczky *et al.*, 2005; Cooper and Goller, 2006; Kao and Brainard, 2006; Teramitsu and White, 2006). It has been suggested that males are in a state of vocal practice when they are alone, and switch to a state of vocal performance when a female is present (Kao *et al.*, 2005; Ölveczky *et al.*, 2005; Kao and Brainard, 2006; Sakata *et al.*, 2008; Sober *et al.*, 2008). This is a well-known example of change in song variability based on social context.

A lot of the research on birdsong in the context of sexual selection has focused on repertoire size as a signal of male quality, without much emphasis on vocal consistency (Byers and Kroodsma, 2009). However, due to the difficulty in performing repetitive motor patterns and the resulting fatigue, vocal consistency has been proposed to be an honest signal of male quality in the context of mate choice and sexual selection (Byers, 2007; Botero *et al.*, 2009; de Kort *et al.*, 2009; Sakata and Vehrencamp, 2012). Several studies have been done to test this hypothesis. In the tropical mockingbird (*Mimus glivus*), there was found to be a significant, positive correlation between vocal consistency and reproductive success (Botero *et al.*, 2009). This hypothesis has also been studied in relation to trill consistency in banded wrens (de Kort *et al.*, 2009) and consistency of spectral features of song in a bout (Byers, 2007). Byers (Byers, 2007) showed that chestnut-sided warbler males that produce more consistent songs produce more extra-pair offspring than males that produce less consistent song. The functional relevance of vocal consistency has been studied by examining how it varies with age, season and social context (Sakata and Vehrencamp, 2012). Hence, vocal consistency could be an important indicator of male quality in the context of sexual selection in songbirds.

The variability in the male zebra finch song is only relevant in mate selection if it is perceptible to the female zebra finches. It has been shown that zebra finches can recognize syllable-level differences in the fine acoustic structure of song. Additionally, they are sensitive to changes in the acoustic structure of vocalizations (Fishbein *et al.*, 2021). Psychoacoustic experiments were conducted to test this, wherein the female bird was required to distinguish between two identical motifs which differed by fine acoustic variation in a single syllable. In this study, a recurring motif was played as the background stimulus, while the test stimulus was created by replacing a single syllable of the original motif with the corresponding syllable from a different motif. It was proposed that the fine changes in acoustic structure of song across different renditions could encode information about the male, which is perceived by the female (Fishbein *et al.*, 2021).

Female zebra finches can not only perceive small changes in different renditions of song, but they seem to exhibit a preference for less variable song. Here, variability is defined by the level of variability in the fundamental frequency across syllables with harmonic stacks. A laboratory study was done on zebra finches, where female zebra

finches were subjected to a preference assay using a three-chamber cage and two speakers on either end of the cage. One speaker played a directed song of the female's mated male, whereas the other speaker played an undirected song of the same male. Preference for one song over the other was inferred by measuring the proportion of time spent near each speaker. Greater proportion of time near the speaker playing directed song would indicate greater preference for directed songs. It was found that females preferred the more consistent directed song over the undirected song (Woolley and Doupe, 2008). They also found that the strength of preference for the directed song over the undirected song was correlated with the magnitude of difference in the syllable consistency between the directed song and the undirected song. This provides indirect evidence that female zebra finches prefer males with lesser variability between different renditions of song.

Although Woolley and Doupe (Woolley and Doupe, 2008) tested the female for its preference for directed song over undirected song and checked for the correlation of preference with the amount of variability in the songs in terms of the fundamental frequency of stacked syllables, they did not provide direct evidence that female zebra finches prefer males with lesser variability in their songs. Also, in the study by Fishbein et al. (Fishbein et al. 2021), they tested if the female zebra finches can identify syllable-level variability between different renditions of song. They did not test if female zebra finches can distinguish whether one set of song renditions is more or less variable than another.

I analysed the variability in the undirected songs of 20 different adult male zebra finches to motivate my study. I hypothesize that female zebra finches can compare the level of variability in the songs of two adult male zebra finches, and prefer males that sing more consistently at the level of the syllables features. To test this hypothesis, I designed two experiments: (1) A preference assay in a three-chamber cage with artificially constructed stimuli of consistent song and variable song played on either side of the cage; (2) An operant conditioning dual choice assay wherein birds were trained to distinguish consistent song stimulus from variable song stimulus. The former examines the bird's natural preferences, while the latter investigates whether the birds can be trained to distinguish between the two stimuli.

# Methods

All experiments were approved by the Institutional Animal Ethical Committee (IAEC), IISER Pune and performed according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi.

### 2.1 Preference Assay

A total of 27 healthy adult female zebra finches were used in this study. The birds were either raised in our lab's colonies or bought locally. These birds were provided with ad-libitum food and received cuttlebone, sprouts (or fresh vegetables) and cooked eggs on a regular basis as supplements. In the bird colony where they were reared, they were maintained on a 14:1 light/dark cycle per day, in a room maintained at 25˚ C. The birds were shifted to individual cages in soundboxes to be kept in isolation 7 days before the start of the experiment. Only a fraction of the 27 birds were responsive, and data from this fraction was analysed further (Table-1).

| Experimental Setup | Total number of female birds used | Number of responsive birds |
|---|---|---|
| Short cage (without lanes) | 4 | 3 |
| Short cage (with partitions) | 8 (4 previously used+ 4 new) | 0 |
| Long cage (with training) | 17 (2 previously used+ 15 new) | 4 |
| Long cage (with pair-bonded birds) | 4 | 2 |

**Table-1 Number of birds used in the preference assay experiment**

#### 2.1.1 Song recording and stimulus preparation

A male zebra finch song bout consists of a series of introductory notes followed by multiple repetitions of a motif. A motif is a distinctive sequence of syllables produced by an individual bird. To prepare the stimulus songs for this study, we recorded the undirected songs of five male birds. Four of these five songs were used as stimuli for

the female bird paired with the corresponding male bird in the paired-bird preference assay experiments. The stimuli for directed song was created using pre-recorded data from the lab. The males were recorded in custom-made sound attenuation boxes, and recordings were done using an omnidirectional microphone (AKG 517) placed on top of the bird's cage. Songs were recorded on a computer using a sound card (44.1 kHz).

The stimulus songs were made by editing the recorded song files in Audacity. The volume of the stimuli was amplified to attain a decibel level of 70-80 dB when played back using a speaker in the choice chamber. The decibel level was checked with a Decibel Meter (Kairos LCD Digital Professional Sound Level Meter). White noise was generated using Audacity, and the file was trimmed to match the duration of the song stimulus. Various types of stimuli were used for different sets of birds in the experiment, a brief summary of which is provided in Table-2. Stimuli were played in a random order.

"Consistent song" stimulus was a song constructed by playing a few Introductory notes, followed by the same motif a fixed number of times (Fig. 1A). For this stimulus, a motif was picked randomly from 10 to 20 different motifs, and was played repeatedly. "Variable song" stimulus was a song constructed by playing a few Introductory notes, followed by different renditions of the motif a fixed number of times (Fig. 1B). For this stimulus, different motifs were picked randomly from 10 to 20 different motifs. An example of different motifs of the same song used in the variable song stimulus is shown in Fig. 2. Fig. 3 shows the amount of variability in a single syllable across different motifs used in the variable song stimulus.

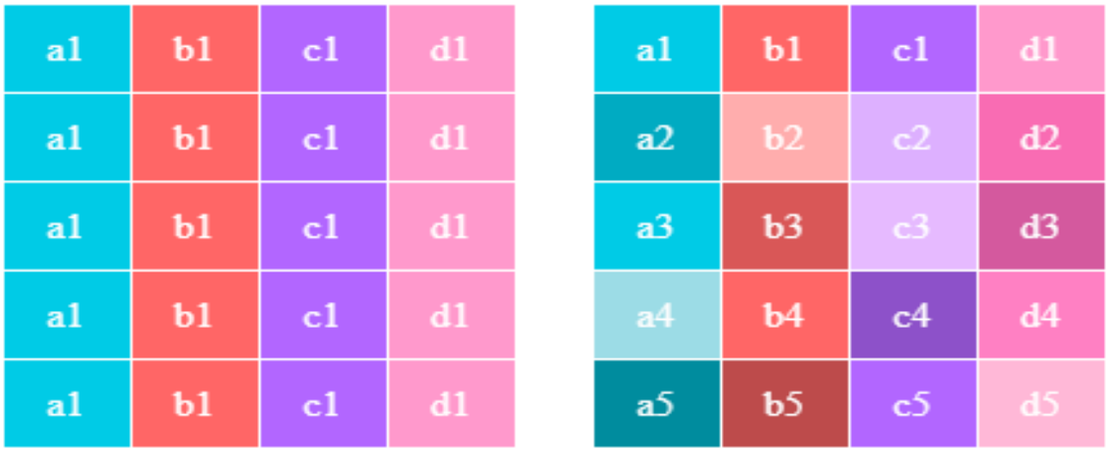



**Figure 1 A depiction of a consistent song stimulus versus a variable song stimulus** A) Consistent song stimulus in which the same motif 'ABC' is played repeatedly. B) Variable song stimulus in which different motifs are played one after the other. The color scheme indicates the syllable level variability across different motifs. Note that the color scheme is purely for representative purposes, and does not translate to the variability observed in real male zebra finch song.

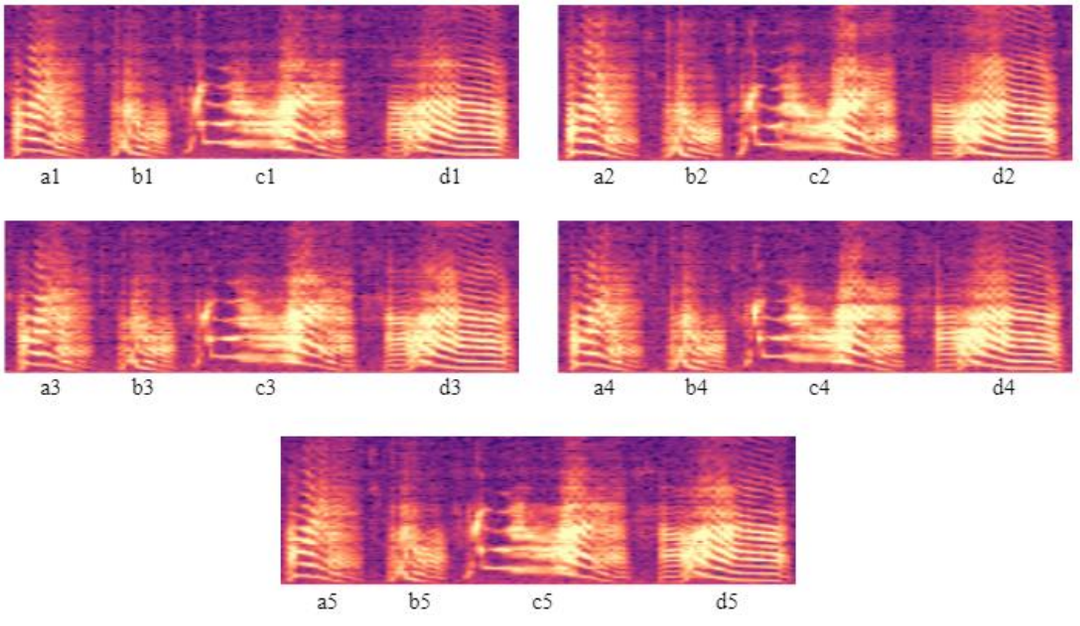



**Figure 2 Spectrograms of 5 different motifs from the undirected song recording of a bird** The syllables are 'a', 'b', 'c' and 'd', and the motif is 'abcd'. The number beside the syllable indicates the motif in which the syllable occurs. Each syllable differs mildly in its spectral features across different motifs (not apparent from the spectrograms)

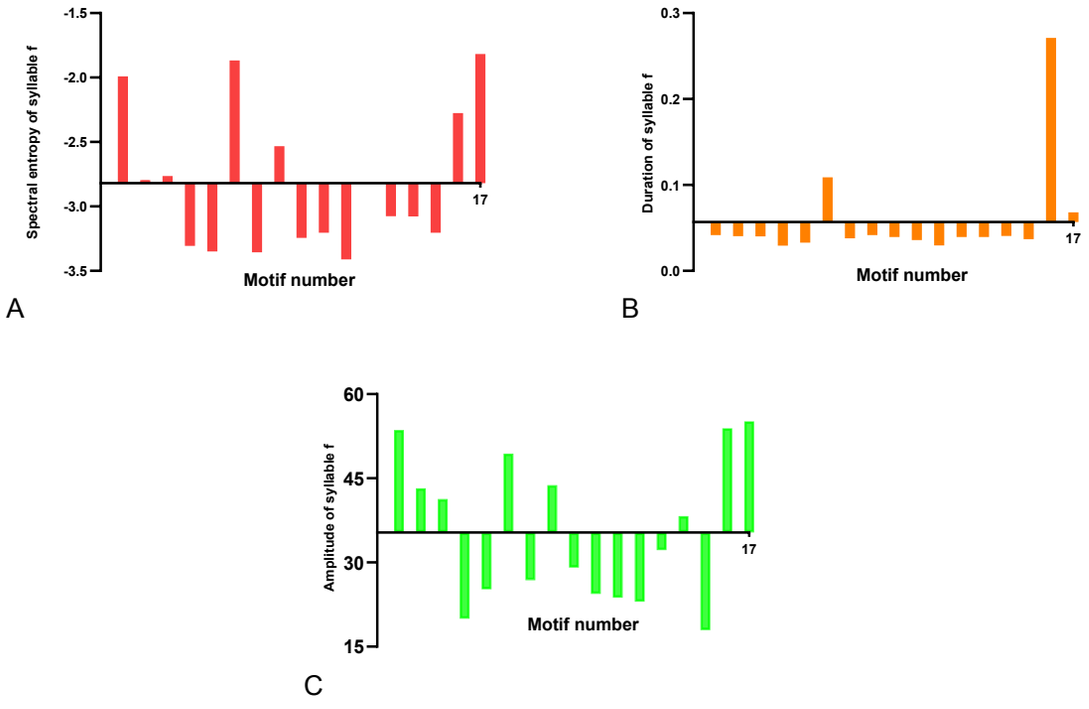



**Figure 3 Syllable level variability across different motifs in the variable song stimulus**. A) Deviation from the mean value of spectral entropy of a syllable across 17 different motifs. B) Deviation from the mean value of duration of a syllable across 17 different motifs. C) Deviation from the mean value of amplitude of a syllable across 17 different motifs. The x-axis intersects the y-axis at the mean value of spectral entropy, duration and amplitude respectively.

### 2.1.2 Experimental design

A schematic of the experimental design is depicted in Fig. 4. The preliminary set of preference assays were done in a wire-mesh short cage (38.5cm × 32cm × 28cm), and the bottom of the cage was lined with a sheet having 6 lanes spaced at 6.3 cm each. Two speakers (Logitech Z120 Stereo Speaker) were placed on either side of the cage, and a light source (Philips 5W LED Cool White Tubelight) was placed at the side of the soundbox, which was later moved to the back to avoid bias. The stimuli were played using a Python code which was custom-written for this experiment. The second set of experiments were done in the same short cage, but with carboard partitions dividing the cage into three equal compartments by width, and with wide rectangular openings in the partitions for the bird to switch compartments. The third and fourth set of experiments were done in a wire-mesh long cage (60cm × 20 cm × 20 cm) which has three compartments with rectangular openings in the partitions for switching compartments. The cages were switched across these experiments to ensure a greater difference in amplitude of the sound between the two side chambers, thereby facilitating easier interpretation of the

results. The experiment was done for four days per bird, but many birds stopped being active after 1 or 2 days of the experiment.

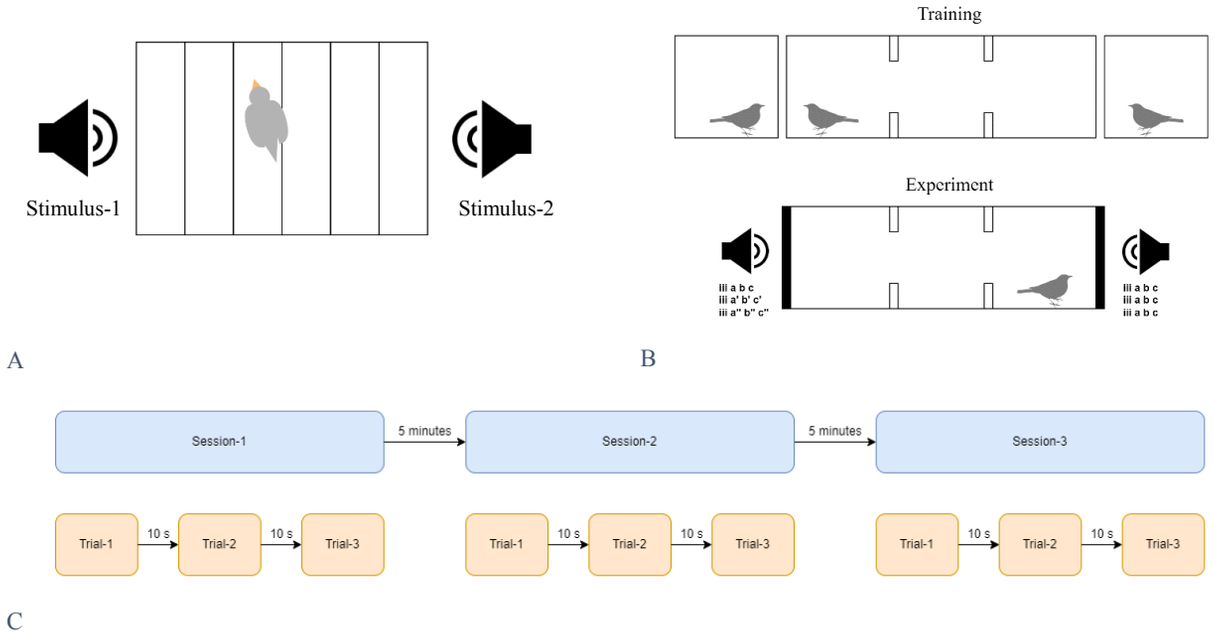



**Figure 4 Experimental setup for the preference assay** A) Short cage experiments with 6 lanes. The lanes were later replaced with partitions dividing the cage into three chambers. B) Long cage experiments with training using two male birds before the experiment. C) Experimental design for the preference assay- each experiment comprises of three sessions, and each session comprises of three trials.

| Experiment Setup | No. of responsive birds: No. of birds used | Total stimulus exposure | Stimulus-1 | Stimulus-2 | Number of sessions and trials per session |
|---|---|---|---|---|---|
| Short cage (lanes) | 3:4 | 217.5 seconds | White noise (3 seconds) | Undirected zebra finch song (3 seconds): 4 INs+ 2 repetitions of motif | 3 sessions, 3 trials. Each trial consists of three iterations of each stimulus. |
| Short cage (partitions) | 0:4 | 217.5 seconds | Variable zebra finch song (3 seconds): Undirected song with 4 INs+ 2 repetitions of motif. | Consistent zebra finch song (3 seconds): Undirected song with 4 INs+ 2 repetitions of motif. | 3 sessions, 3 trials. Each trial consists of three iterations of each stimulus. |

| | | | | | |
|---|---|---|---|---|---|
| | 0:4 | 217.5 seconds | Zebra finch song (3 seconds): Undirected song with 4 INs+ 2 repetitions of motif. | Java finch song (3 seconds): Undirected song with 2 INs+ motif. | 3 sessions, 3 trials. Each trial consists of three iterations of each stimulus. |
| Long cage with training | 2:8 | 217.5 seconds to 570 seconds | Test: Consistent zebra finch song (3 seconds): Undirected song with 4 INs+ (2 to 5) repetitions of motif.<br><br>Positive control: White noise (3 seconds)<br><br>Negative control: Consistent zebra finch song (3 seconds): Undirected song with 4 INs+ (2 to 5) repetitions of motif. | Variable zebra finch song (3 seconds): Undirected song with 4 INs+ (2 to 5) repetitions of motif.<br><br>Consistent zebra finch song (3 seconds): Undirected song with 4 INs+ (2 to 5) repetitions of motif.<br><br>Consistent zebra finch song (3 seconds): Undirected song with 4 INs+ (2 to 5) repetitions of motif. | 3 sessions, 3 trials. There is one session of test, one session of positive control, and one of negative control. The order of each session is randomized. Each trial consists of three iterations of each stimulus. |
| | 1:4 | 1063 seconds | Consistent zebra finch song (3 seconds): Undirected song with 4 INs+ 10 repetitions of motif. | Variable zebra finch song (3 seconds): Undirected song with 4 INs+ 10 repetitions of motif. | 3 sessions, 3 trials per session. Each trial consists of three iterations of each stimulus. |
| | 1:4 | 153 seconds | Consistent zebra finch song (3 seconds): **Directed** song with 4 INs+ 10 | Variable zebra finch song (3 seconds): **Directed** song with 4 INs+ 10 repetitions of motif. | 3 sessions, 3 trials per session. Each trial consists of **one** iteration of each stimulus. |

| | | | | | |
|---|---|---|---|---|---|
| | | | repetitions of motif. | | |
| Long cage with pair-bonded birds | 2:4 | 250 seconds | Consistent zebra finch song (3 seconds): Undirected song of pair-bonded male with 2 to 4 INs+ 10 repetitions of motif. | Variable zebra finch song (3 seconds): Undirected song of pair-bonded male with 2 to 4 INs+ 10 repetitions of motif. | 3 sessions, 3 trials per session. Each trial consists of one iteration of each stimulus. |

**Table-2 Stimuli used for each preference assay experiment.** The stimuli were changed with each set of experiments in an attempt to maximize the response of the birds.

### 2.1.3 Long cage experiments- Acclimation and training

In the long cage experiments with training, the birds were given training on 4 alternate days in a week. The training involved placing two male birds on either side of the long cage for a 10-15 minute period for each female bird that is put in the long cage. The males were changed every day, but the same males were used for all the female birds in that experimental set on any given day. The idea behind this was that the females would start actively moving in the long cage. At the end of the training period, I expected the birds to be more motivated to respond and move between compartments when the speakers are placed on either ends of the cage.

For the experiment, the ends of the long cage were covered with a sheet, and a speaker was placed on either end. The experiment mainly consists of three sessions separated by 5-minute intervals. Each session consists of three trials separated by intervals of 10 seconds each. Before the start of each set of experiments, the amplitude of the playback from each speaker was measured in each chamber using a Decibel Meter in order to adjust the volume on the laptop accordingly, and also to ensure that the playback from both the speakers was equal in amplitude. Typical decibel values from both the speakers for a stimulus is shown in figure-5. The stimuli were modified across the different experimental sets of birds in order to try to maximize the responsiveness of the female birds.

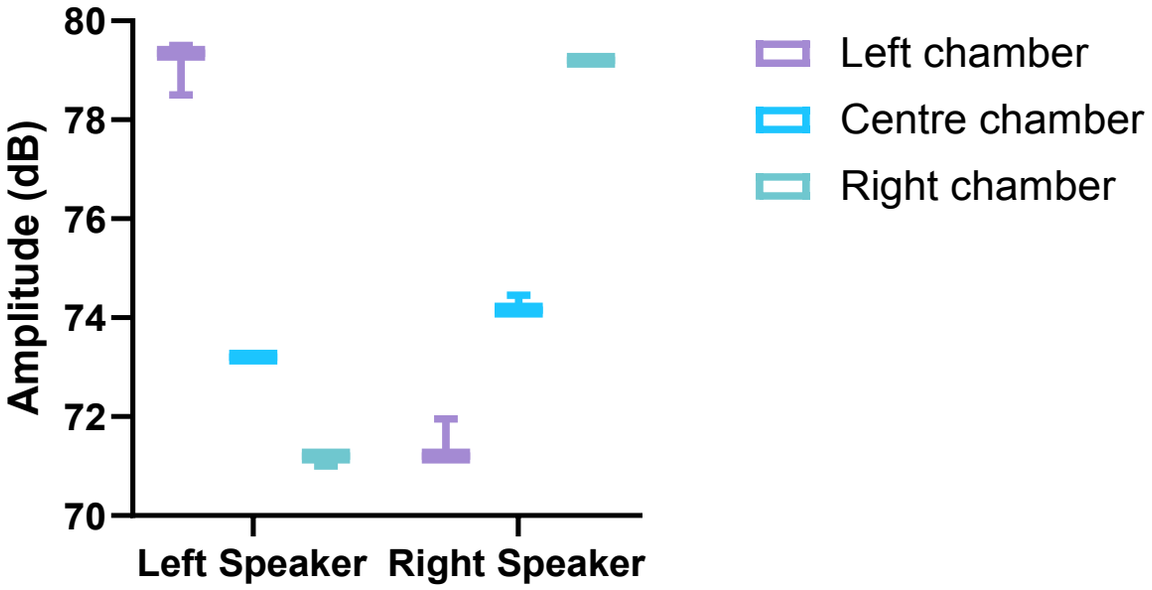



**Figure 5 Amplitude of the playback form each speaker in the three chambers of the long cage**. This is a box plot of the amplitude values in each chamber when there is a playback from either one of the two speakers. The amplitude value is maximum at each of the side chambers for the playback of the corresponding speaker, and minimum for the playback of the other speaker.

### 2.1.4 Long cage experiments- Pair bonded birds

To determine whether prior exposure and bonding to a male would improve the responses in the preference assay, I used pair-bonded female birds in the last set of experiments in the long cage. Before the experiment, the individual females were isolated and placed in the same cage as a male bird whose song they responded to. The male and female were kept together in the same cage for a week, and their behaviour was noted every day. At the end of a week, it was observed that the male and female sat together and spent a lot of time in close physical proximity. Hence, I regarded them as pair-bonded, and after the week of pair-bonding, I isolated the female bird for a period of 5 to 7 days in a sound attenuating box. The undirected song of the pair-bonded male bird of each female bird was recorded, and the stimuli were made using these songs. After the isolation period, the birds were directly introduced to the long cage, and the experiment was done. No training was done in this case.

Another set of experiments were done on all the responsive birds from the long cage experiments, where the sides that played consistent and variable songs were switched. Experiments were repeated with individual birds after a defined period of time, which varied from a few days to 5 months. Only 2 out of 6 birds were responsive in this experiment.

### 2.1.5 Video scoring

The experiments were conducted in custom-made sound attenuating boxes placed in rooms away from the bird colonies, with minimal human noise in the surroundings. All video recordings were done using Logitech Webcam C270 HD with frame rate of 30 fps. Videos were analysed for the duration of time spent in each compartment, and the number of compartment switches using BORIS (Friard and Gamba, 2016). Manual checking was also done for the initial set of experiments. Manual checking involved recording the precise time points at which the chamber transitions occurred, followed by the manual calculation of the time spent by the birds in each chamber. In the long-cage experiments, the duration of time spent by the bird in each chamber, switches between chambers, and the number of calls given by the bird were scored according to the stimuli played by the speaker.

### 2.1.6 Data analysis and Statistical analysis

If the bird exhibited more than three chamber transitions over the whole experimental period of three sessions, I considered data from that bird for further analysis. All birds with less than three chamber transitions over the whole experimental period (3 sessions) were excluded from further analysis. The values of time spent in each chamber, the number of times the bird hops into a particular chamber (number of occurrences), the number of chamber transitions exhibited by the bird when a given stimulus is played (bird activity levels) and the number of calls given by a bird were considered as different measures of preference. These values were calculated using the BORIS software. I normalized the data by taking the proportion of duration spent in the chamber where consistent song was played, over the duration spent by the bird in both the chambers where consistent and variable song stimuli were played. I decided to exclude the duration spent in the central chamber as it does not add to interpreting the preference for one stimulus over another. I also excluded the time

spent by the bird in either of the two chambers during the inter-stimuli interval of 10 seconds, and also during the inter-session interval of 5 minutes, since the birds were largely inactive and did not switch chambers during these periods. A similar approach was taken for the other measures of preference. Table-3 lists the formulae used to calculate these preference measures.

All plots were made using Python or Graphpad Prism 9 Software. Statistical tests and correlation tests were done using Graphpad Prism 9 Software. Wilcoxon signed rank test was used to test for the significance of the preference for consistent or variable song using the different measures of preference. Spearman correlation coefficient was used to test for correlation between the different measures of preference using the preference indices.

| S. no. | Description | Formula | Key |
|---|---|---|---|
| (1) | Proportion of time spent in chamber where consistent song is played | $\frac{[\mathrm{C(t)} + \mathrm{V(t)}]_{consistent}}{[\mathrm{C(t)} + \mathrm{V(t)}]_{consistent} + [\mathrm{C(t)} + \mathrm{V(t)}]_{variable}}$ | $[]_{consistent}$ = Consistent song chamber<br>$[]_{variable}$ = Variable song chamber<br>C(t) = Time spent in a chamber when consistent song is played<br>V(t) = Time spent in a chamber when variable song is played |
| (2) | Proportion of time spent in chamber where variable song is played | $\frac{[\mathrm{C(t)} + \mathrm{V(t)}]_{variable}}{[\mathrm{C(t)} + \mathrm{V(t)}]_{consistent} + [\mathrm{C(t)} + \mathrm{V(t)}]_{variable}}$ | |
| (3) | Proportion of number of visits to the chamber where consistent song is played | $\frac{[\mathrm{C}(n) + \mathrm{V}(n)]_{consistent}}{[\mathrm{C}(n) + \mathrm{V}(n)]_{consistent} + [\mathrm{C}(n) + \mathrm{V}(n)]_{variable}}$ | C(n) = Number of hops into a chamber when consistent song is played<br>V(n) = Number of hops into a chamber when variable song is played |
| (4) | Proportion of number of visits to the chamber where variable song is played | $\frac{[\mathrm{C}(n) + \mathrm{V}(n)]_{variable}}{[\mathrm{C}(n) + \mathrm{V}(n)]_{consistent} + [\mathrm{C}(n) + \mathrm{V}(n)]_{variable}}$ | |
| (5) | Proportion of bird activity level when | $\frac{C(No.of\ transitions)}{C(No.of\ transitions) + V(No.of\ transitions)}$ | C(No. of transitions) = |

| | | | |
|---|---|---|---|
| | consistent song is played | | Number of chamber transitions during consistent song<br><br>V(No. of transitions) = Number of chamber transitions during variable song |
| (6) | Proportion of bird activity level when variable song is played | $\frac{V(No.of\ transitions)}{C(No.of\ transitions)+V(No.of\ transitions)}$ | |
| (7) | Proportion of number of calls when consistent song is played | $\frac{C(No.of\ calls)}{C(No.of\ calls)+V(No.of\ calls)}$ | C(No. of calls) = Number of calls given by the bird during consistent song<br><br>V(No. of calls) = Number of calls given by the bird during variable song |
| (8) | Proportion of number of calls when variable song is played | $\frac{V(No.of\ calls)}{C(No.of\ calls)+V(No.of\ calls)}$ | |
| (9) | Preference index for time spent in consistent song chamber | (1) – (2) | |
| (10) | Preference index for number of visits to consistent song chamber | (3) – (4) | |
| (11) | Preference index for activity level during consistent song | (5) – (6) | |
| (12) | Preference index for number of calls during consistent song | (7) – (8) | |

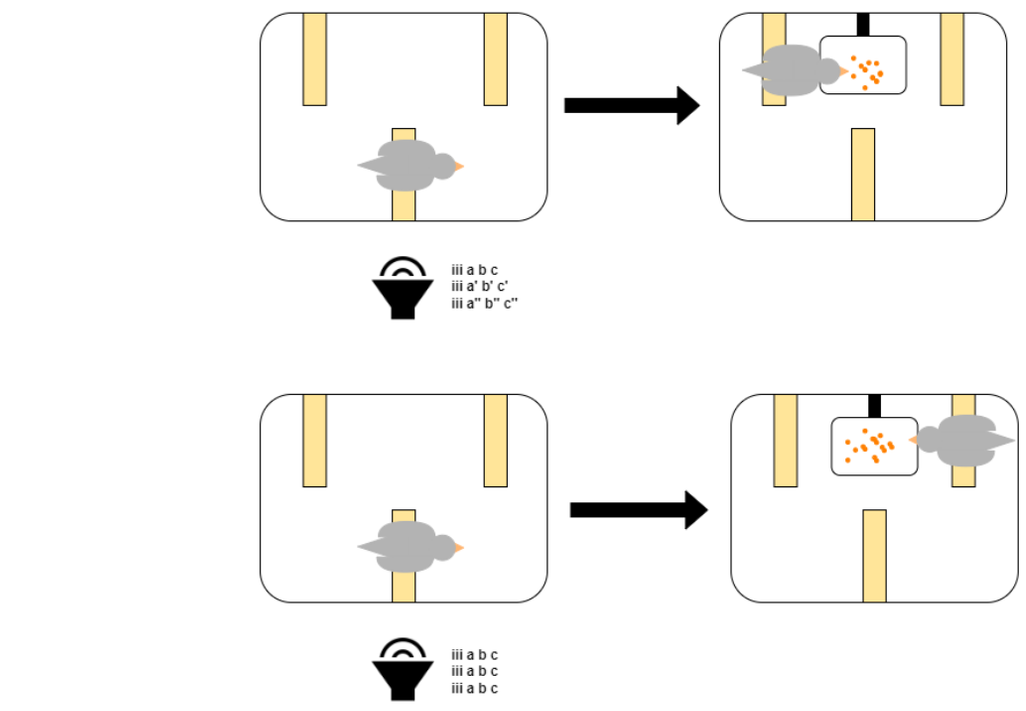
**Table-3 List of formulae used in analysing preference for consistent or variable song**

## 2.2 Dual choice classification task experiment

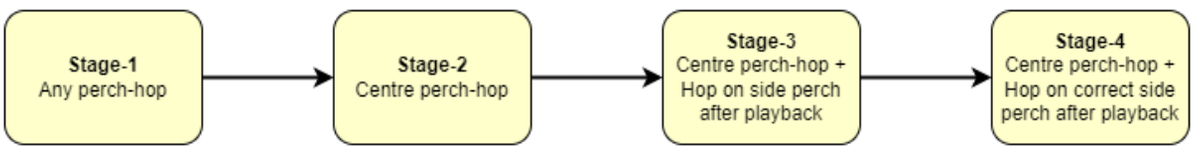
### 2.2.1 Experimental Design

Fig. 6A depicts the experimental design for the dual choice classification task. There are three perches in the setup: Two on either sides of the cage and one in the centre. The bird hops on the centre perch to initiate a trial. In a trial, either variable

song or consistent song is played by a speaker. The task is to correctly classify the song as variable or consistent and hop on the correct side perch associated with the song to get a food reward. The training for this task is accomplished using four stages of training (Fig. 6B). In the first stage, the bird is rewarded for hopping on any one of the three perches. In the second stage, the bird is rewarded only if it hops on the centre perch. In the third stage, the bird is rewarded if it hops on the centre perch and then hops on any one of the side perches after the playback is complete. In the third stage, the playback stimulus comprises of a single motif rather than the final stimulus of a variable or consistent song. This approach was employed to prevent any inadvertent associations between the two perches and the playback stimulus from being formed by the birds. In the last stage of training, the bird needs to hop on the centre perch to initiate a trial, and then hop on the correct side perch after playback stimulus (variable or consistent song) is complete.




A




B

**Figure 6 Experimental design for the dual choice classification task experiment. A**) The bird hops on the centre perch to initiate a playback. It gets a food reward if it correctly classifies the song as consistent or variable. B) The different stages of training used to train the birds.

### 2.2.2 Experimental Setup

Three perches, equipped with IR beams to detect a bird hopping onto a perch, were fitted in a cage (Fig. 7A). Two perches were fixed on either side of the cage while the last perch was fixed at the front of the cage. A feeding system was setup opposite to the last perch, between the two side perches. A webcam was fixed at the top of the cage. An Arduino was connected to the IR beam circuitry and the motor which delivered food reward. The Arduino was powered by a laptop and controlled using a MATLAB script. The setup was placed in a room with minimal human disturbance. When a bird hops onto a perch, the data is recorded into a text file generated by MATLAB. Upon a bird's hopping onto a perch, the corresponding data regarding the perch hops made by the bird is duly noted and saved into a MATLAB-generated text file.

### 2.2.3 Feeding system for reward

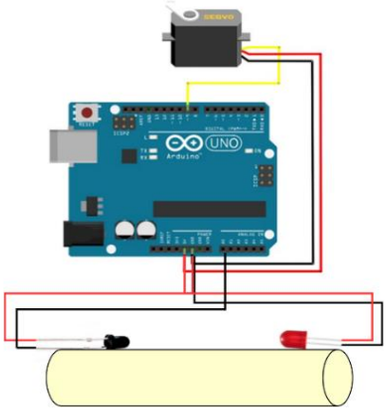
The initial design of the reward was that of seeds being dropped from the feeding cup into the seed cup placed below. This would happen whenever the bird performed the task correctly. To build this, I used a servo motor which rotates the lid of a can to drop seeds from the can into a seed cup placed below it. I tried to train the birds using this setup, but I soon discovered that the birds could hop on the different perches randomly (and have the seed cup fill up), and then eat the seeds since there was no mechanism to regularly clear out the seeds in the seed cup. Thus, I made a different feeding system in which the seed cup moves into the cage as a reward, stays in the cage for a few seconds (7s), and then moves out of the cage. In order to build this, I used another servo motor which was connected to the seed cup by means of wooden sticks, such that the sticks unfold to move the seed cup along a rail when the motor rotates by a specific angle, and refold to move the seed cup back along the rail when the motor rotates back by the same angle. Fig. 7B shows the experimental setup with the modified feeding system.

### 2.2.4 Circuit

Fig. 7C depicts a schematic diagram of the circuit. IR photodiode receiver-transmitter pairs were fitted onto the perches to detect and monitor hops onto the perches. All the connections in the circuit were soldered onto a circuit board, which was then connected to the Arduino Uno microcontroller board. The IR transmitters were connected to the 5V and ground of the Arduino for a 5V voltage. The IR transmitters were all connected in series through a 100 ohms resistor. The negative legs of the IR receiver photodiodes were connected to the digital pins of the Arduino through 3.3k ohms resistors. The digital pins of the Arduino were configured to provide 5V voltage to the photodiodes. The positive legs of the photodiodes were connected to ground. The motor (Sg90 Micro Servo Motor 9G Rc) was connected to the 5V, ground and a digital pin on the Arduino.

A

B




C

**Figure 7 Experimental setup for the dual choice classification task experiment** A) Initial setup with the food reward (seeds) being dropped into the seed cup when the bird does its task correctly. B) Modified setup with a seed cup moving in the cage for a few seconds when the bird does its task correctly. C) Circuit for the operant conditioning setup. The circuit mainly consists of IR transmitters and receivers, and a servo motor connected to an Arduino.

# Results

## 3.1 Variability in the male zebra finch song

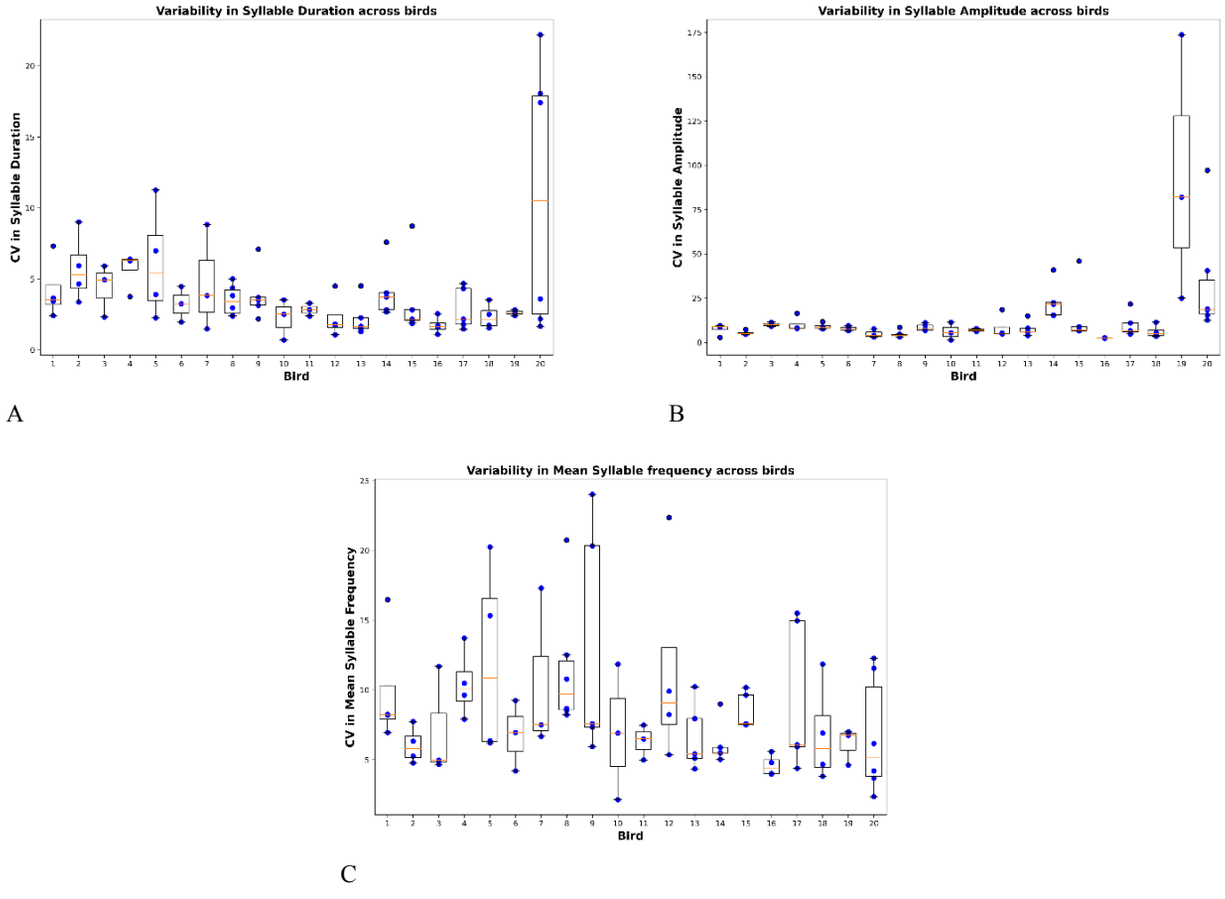



**Figure 8 Variability in male zebra finch song** Coefficient of variation in A) Syllable duration B) Amplitude C) Mean Syllable Frequency across 20 adult male zebra finches. Different blue dots represent different syllables present in the motif of that bird's song. The median value of CV of each bird is indicated by the orange lines in the box plots.

To determine the range of syllable-specific variability that exists across birds, I took the annotated undirected song data of 20 birds from the lab and plotted the coefficient of variation of the syllable duration, syllable amplitude, and mean syllable frequency of every syllable for each of these 20 birds. The data comprised of 20 to 30 labelled undirected song motifs per bird, stored in text files generated by a custom written MATLAB script used in the lab. Different birds have different levels of variability, and within each bird, different syllables had different levels of variability (Fig. 8). These results show that the song variability differs considerably across birds

and could represent a basis for female mate choice. Therefore, it was important to ask whether female zebra finches prefer more variable or more consistent songs.

The amount of variability seen in a specific bird's song also depends on which measure is used to calculate variability (CV in mean syllable frequency, syllable amplitude, syllable duration etc). I checked how correlated these different measures are in providing a value of variability for each bird by taking the mean CV value for all the syllables of a bird for each measure. Mean CV in mean syllable frequency and Mean CV in syllable duration are mildly correlated (Spearman r= 0.284). Mean CV in syllable duration and mean CV in syllable amplitude are also mildly correlated (Spearman r= 0.329). Mean CV in mean syllable frequency and mean CV in amplitude are uncorrelated (Spearman r= 4.51e-003). Thus, depending on which syllable parameter is considered, the extent of variability exhibited by a bird could be different.

### 3.2 Preference assay- Preliminary Experiments3.2.1 Birds prefer zebra finch song over white noise in this setup

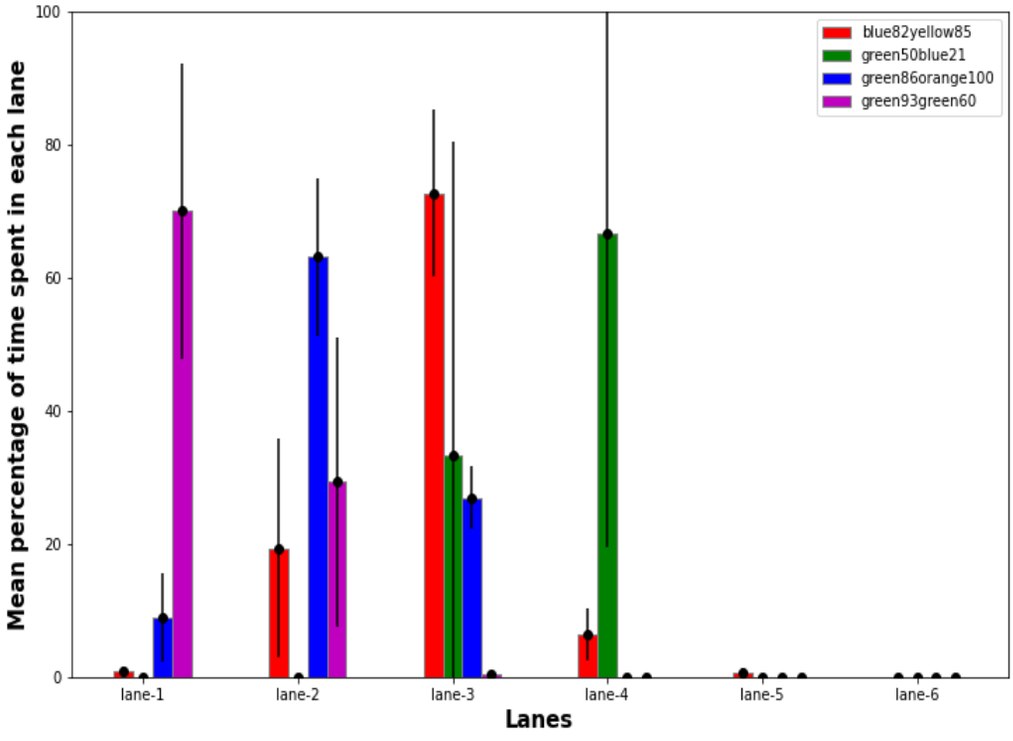



**Figure 9 White noise versus zebra finch song- Short cage setup** Bars indicate mean percentage of time spent by the birds in the six lanes over three days of experiment (3 sessions per day). Zebra finch song was played on the side near lane-1, whereas white noise was played on the side near lane-6. The error bars indicate the standard deviation.

In the first set of preference assays in the short cage, I marked 6 lanes at the bottom of the cage and performed a control experiment where I played zebra finch song on one side of the cage and white noise on the other side of the cage. This was used to determine whether the setup worked to elicit a side-preference from the birds. Specifically, I wanted to check if the width of the cage is sufficiently large to see a side preference from the birds when two different stimuli are played on either side of the cage, and also to check the extent of side-preference that can be observed. Birds spent most of their time on the side where song is played (Fig. 9).

I isolated the same birds for a few days after the initial experiment and then tested the birds for their preference for consistent song or variable song stimuli in the same setup. Irrespective of which stimuli was played on either side, I observed a preference for the side closer to lane-1, just like in Fig. 9 (Fig. 10). This suggested that birds had already developed a side-preference from the previous control experiment and this side-preference was now independent of the stimulus played.

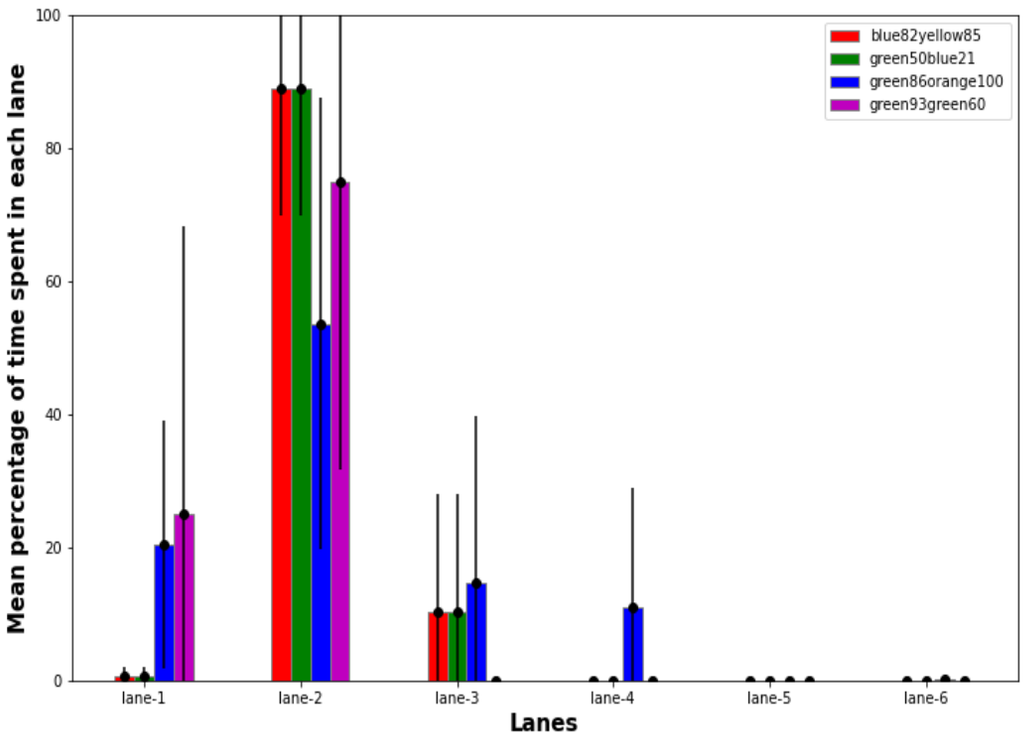



**Figure 10 Variable versus consistent song- Short cage setup** Bars indicate mean percentage of time spent by the birds in the six lanes over four experimental days (3 sessions per day). In two of the sessions, variable song was played near lane-1 and consistent song was played near lane-6. The sides that played variable and consistent song were switched in the other two sessions. The error bars indicate the standard deviation.

### 3.2.2 Birds are not motivated to switch compartments in the setup

To avoid such side preferences, I used a new set of birds for every preference assay experiment conducted thereafter. I modified the setup by placing two partitions at equal spacing along the width of the cage to mimic a three-chamber cage. I speculated that this would help in interpreting the side preference of the bird better than having 6 lanes with no partitions where the bird can freely roam around the whole cage. I tested a new set of female birds for their preference of zebra finch song over java finch song, with the expectation that they would prefer the song of their own species over that of the java finch. The motivation for this experiment was to test the extent of side-preference that the birds exhibited. However, throughout the experimental period, the birds were not active and they exhibited at most two chamber-transitions (hopping from one chamber into another). Hence, I decided to modify the experimental procedure by attempting to increase the motivation of the birds to switch chambers.

## 3.3 Long Cage Experiments

### 3.3.1 Birds show a general increase in activity with training and acclimation

In the long-cage three-chamber setup, I put the birds through a training phase wherein they would be put in the setup with two male birds placed on either side of the cage. The motivation for doing this training phase was to make the birds accustomed to male birds being present on either side of the three-chamber cage. After the training phase, the birds were put through an experimental phase where the sides of the three-chamber cage were covered with a sheet, and speakers which played the opposing stimuli were placed on either side. Bird activity, measured by the number of chamber transitions, increased from the first to the fourth day of training (Fig. 11). However, a statistical test on this data (ANOVA: $p=0.0909$, followed by Dunnett's multiple comparisons test) indicates that there is no significant difference between the activity level on day-1 and day-4 (p-value= 0.1583). Bird activity level on day-3 is also not significantly different from day-1 (p-value= 0.1884).

However, the activity level on day-2 is significantly higher than the activity level on day-1 (p-value= 0.0411). Thus, although training did help in making the birds accustomed to the setup and made them more active during the preference assay, these results suggest that 1-2 days of training and acclimation would suffice.

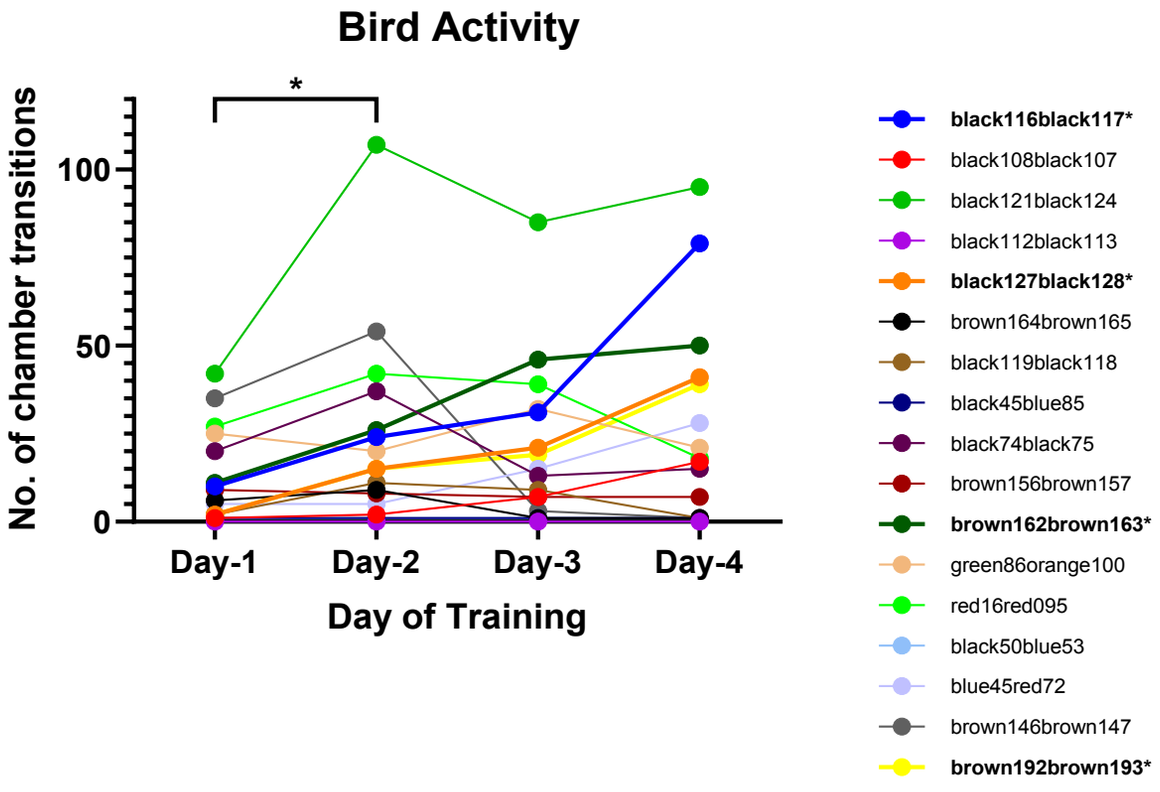



**Figure 11 Activity of the bird over four days of training** The circles represent birds, and each colour represents one bird. The same bird is connected by a line across the 4 days of training. Bird names written in bold and marked with an asterix are the ones that were active during the experiment after training. There is a significant increase in bird activity from day-1 to day-2, and this is marked with an asterix in the plot.

### 3.3.2 Birds which sampled all chambers and showed increased activity over training were used in the preference assay

The birds which showed a gradual increase in activity from the first day of training to the last day of training were typically found to be responsive and active in the preference assay. Some birds showed a decline in their activity level over training, and some birds remained inactive throughout the training phase. Only birds which had at least visited all the three chambers once were selected for the preference assay. Fig. 12 shows the percentage of time spent by the birds in each of the three

chambers in the first and last days of training. The expected outcome was for the birds to spend an equal amount of time in the side chambers by the fourth day of training, resulting in a lower absolute difference between the time spent in the right

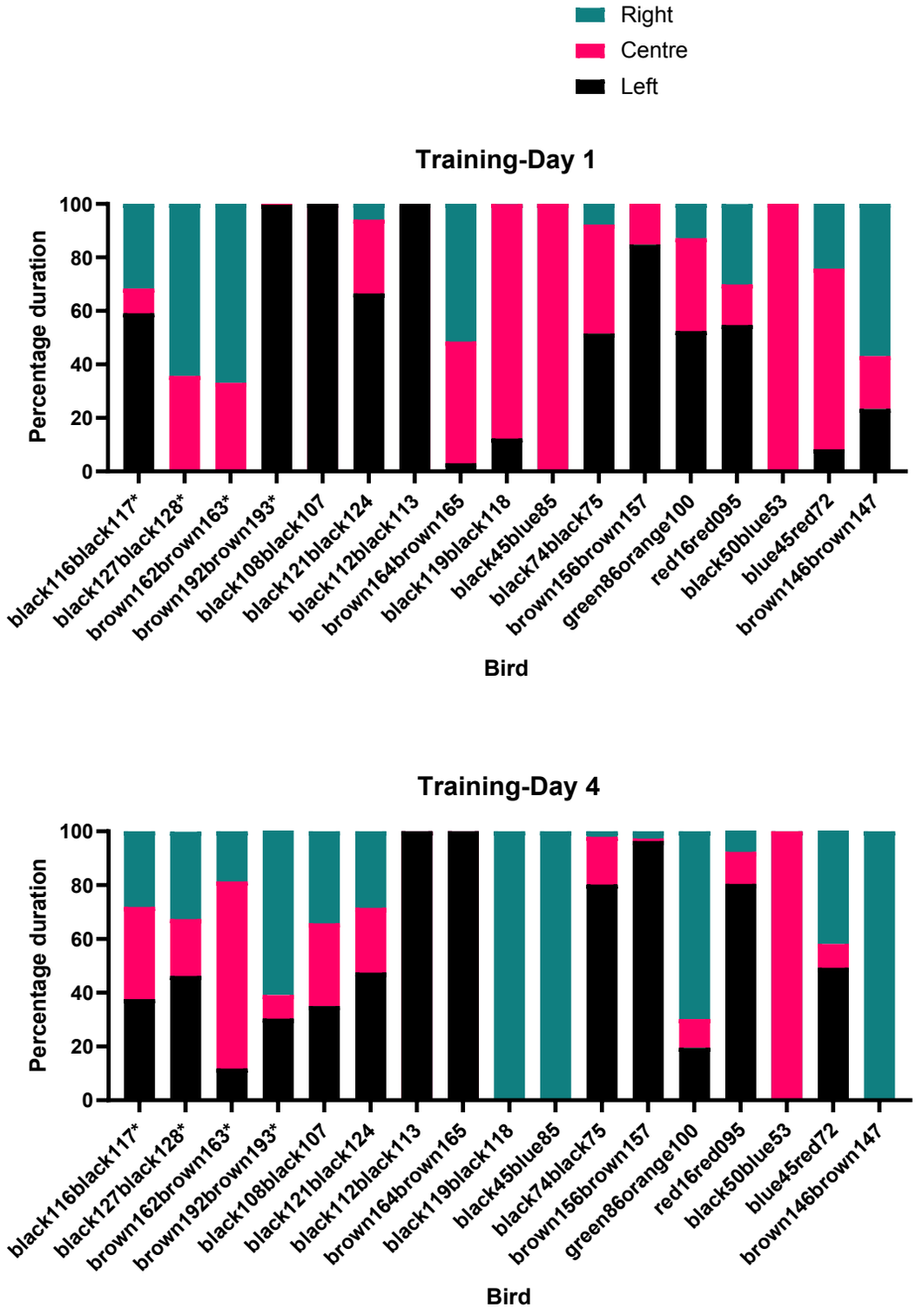



**Figure 12 Training Day-1 and Day-4** Time spent by the bird in each chamber (left, centre or right) in the first and last training sessions. The asterix* indicates birds which were active in the experimental sessions after training.

and left chambers on day-4 compared to day-1. While 41% of the birds adhered to this expectation, an equal proportion of birds spent time more equally on day-1

compared to day-4. Moreover, 18% of the birds remained inactive throughout the training, and did not visit more than one chamber. Thus, training did not help in making the birds spend equal amounts of time in the side chambers prior to the preference assay.

### 3.3.3 Preference assay using controls could affect the response of the birds towards test stimuli

In the first two sets of preference assays that I did in the long cage (n=8; 2 responsive birds), I used controls as stimuli along with the test stimuli. For both the sets of experiments, I conducted three sessions: Positive Control (Zebra finch song on one side, white noise on the other), Negative control (Same song on both sides) and Test (Consistent song on one side, variable song on the other). The order of the sessions was randomized, but the sides which played consistent and variable song stimuli were kept constant throughout the experiment for a given bird. In the first set of experiments (n=4; responsive bird: black116black117), the white noise was always played on the same side as consistent song. Hence, this control was used as a positive control. However, I speculated that the bird could develop a side-preference based on just the positive control alone. Hence, in the second set of experiments (n=4; responsive bird: black127black128), white noise and song in the control were randomly played on different sides throughout the experiment. As the song and white noise were played randomly from both sides, the stimuli did not serve as a positive control since the birds were expected to spend equal time in the side chambers over all the experimental sessions.

Fig. 13 shows the results of the experiment, along with hypothetical expected outcomes of the experiment for the two birds. The hypothetical outcome for the test stimuli (consistent song versus variable song) was made in accordance with my hypothesis that the birds prefer consistent song. Note that the percentages in the hypothetical outcomes were chosen arbitrarily, and only the broad trends in the plot reflect the expected result.  As can be seen from figure-12, black116black117 exhibits some trends that were expected, but the error bars are large and overlapping, making interpretation difficult. Black116black117 spent more time on the side of the cage associated with song (in the positive control session) and variable song (in the test session), as compared to the other side of the cage. Black127black128 does not exhibit the expected trends, and it spent most time in the

chamber associated with variable song, and this side preference was seen during the control sessions as well.

For further experiments, I decided to replace the control sessions with additional test sessions. Thus, the experiments would consist of three test sessions, and no controls. This modification was made since I speculated that having two control sessions could decrease the response of the bird to the test session, and could also bias the bird towards one side. Having three test sessions in one experiment would also increase the amount of data for the test stimuli. Since my sample size is low, I used the data from the test sessions (and not the controls) from these birds for further analysis.

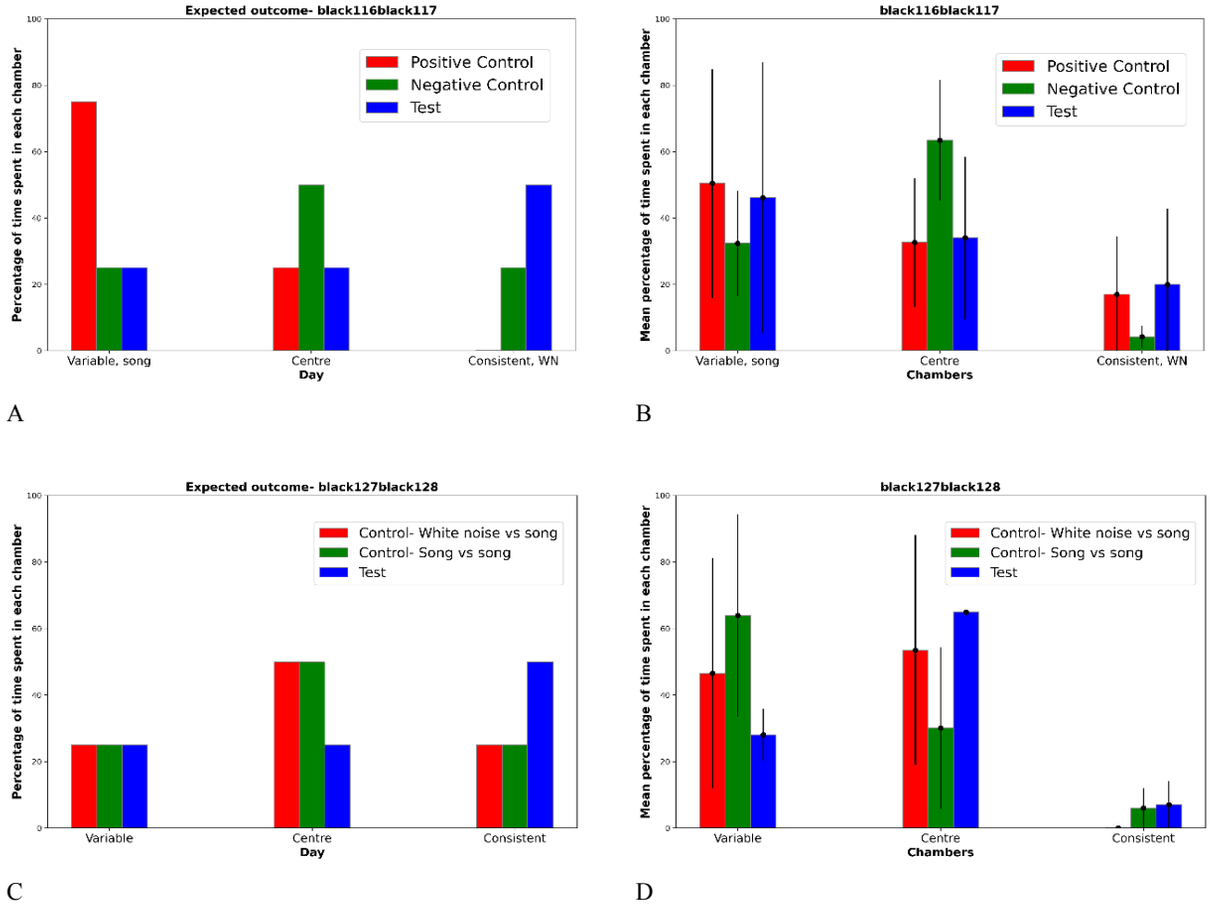



**Figure 13 Expected outcomes and results of the preference assay with controls** A) Expected outcome of black116black117. 'Variable, song' chamber indicates the chamber where variable song was played in the test session and song was played in the positive control. 'Consistent, WN' indicates the chamber where consistent song was played in the test session and white noise was played in the positive control. B) Results of the preference assay for black116black117- Mean percentage of time spent in each chamber for positive control, negative control, and test sessions over 4 days of experiment C) Expected outcome of black127black128. 'Variable' and 'Consistent' chambers indicate the chambers where variable and consistent song were played respectively in the test session. The sides which played white noise and song were randomized in the positive control. D) Results of the preference assay for black127black128- Mean percentage of time spent in each chamber for the two controls sessions and the test session over 2 days of experiment. Error bars depict the standard deviation.

### 3.3.4 Birds spend an unequal amount of time in each of the side chambers

All the birds (with the exception of brown162brown163) spent a much bigger amount of time in one of the side chambers as compared to the other (Fig. 14) over the whole experimental period. This suggests that birds do have a side preference, and the side preference is different across birds However, whether this side preference is due to the different stimuli (consistent song versus variable song) played on either side of the long cage cannot be inferred directly from this data.


**Figure 14 Total duration spent in each chamber- All birds** Total duration Total duration spent by the birds in each chamber over all the experimental sessions.

### 3.3.5 Birds show variable trends in the time spent in each chamber for the corresponding stimulus

Fig. 15 shows the time spent for each stimulus in the three chambers. The expected trend is that, of the total time spent by the bird in a side chamber, most of the time in that chamber is spent when the stimulus corresponding to that side is played. If this trend is followed, we can then make conclusions about the preference of the bird for a specific stimulus based on the total duration spent in each side chamber.

Some birds (black116black117- LV RC and black124black125- LV RC) hardly visited one of the side chambers during the experiment, but showed a trend in preference, if only one side chamber and the central chamber are considered. Some birds

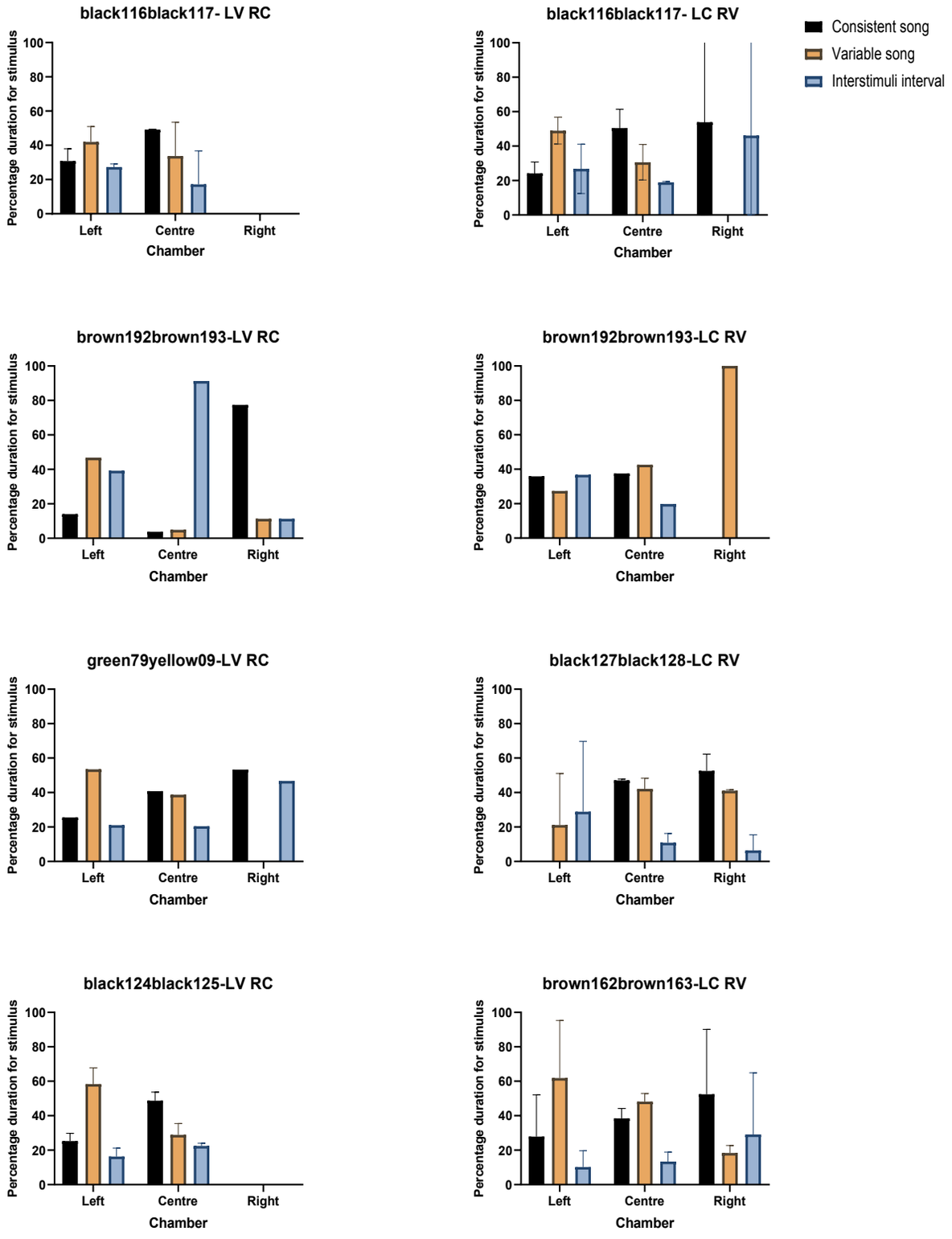



**Figure 15 Mean percentage of time spent in a chamber for each stimulus**. Bars indicate the mean percentage of time spent by the bird for each stimulus played (consistent song, variable song, or interstimuli interval- no song) in the left, centre and right chambers over 1 to 3 experimental days. All the bars in each chamber add up to 100%. 'LV RC' indicates that variable song was played on the left and consistent song was played on the right, whereas 'LC RV' indicates that consistent song was played on the left and variable song was played on the right. Error bars indicate the standard deviation.

(green79yellow09- LV RC, brown192brown193- LV RC) showed the expected trend whereas other birds (black116black117- LC RV, black127black128- LC RV, brown162brown163- LC RV) did not show the expected trend. For example, in the case of green79yellow09- LV RC, variable song was played on the left side and consistent song was played on the right. The bird spent more time in the left chamber when variable song was played than when consistent song was played, and it spent most time on the right side when consistent song was played. The birds which did not spend most time on one side when the stimulus corresponding to that side was played, could have delayed responses to the stimuli or could be switching chambers randomly. Since my sample size is very small, I decided to use all the birds' data to analyse preference, and not just the ones which show the correct trend. To make my analysis more rigorous, I also considered other measures of preference such as the number of calls given by the birds for a specific stimulus.

### 3.3.6 No significant preference using duration as a measure for preference

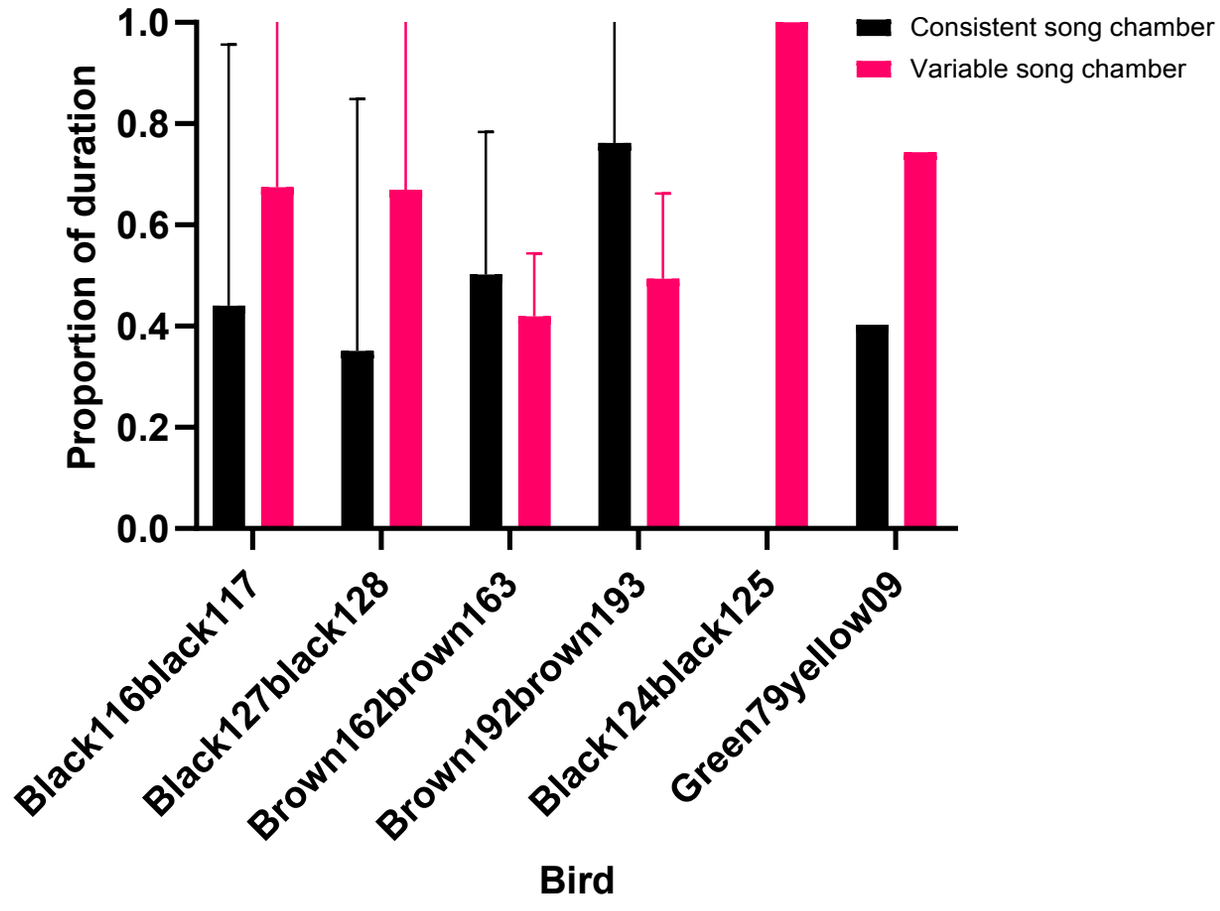



**Figure 16 Proportion of time spent in consistent and variable song chambers.** Bars indicate the proportion of duration spent by the bird in the chambers near where consistent and variable songs were played (left or right chambers). The error bars indicate the standard deviation.

The proportion of duration spent by the bird in the chambers near where consistent song and variable song were played (labelled as “Consistent song chamber” and “Variable song chamber” in Fig. 16) was calculated using the formulae given in Table-3). A Wilcoxon signed rank test was performed on the data, and no significant difference between the two groups was observed (p-value = 0.2188). Thus, in terms of the time spent in the chambers near where either of the two stimuli were played, the birds do not exhibit a significant preference for one of the stimuli over the other.

### 3.3.7 No significant preference using number of visits as a measure for preference

A similar approach was taken to calculate the proportion of number of visits to a specific chamber during a specific stimulus (Fig. 17; see Methods for formula). A

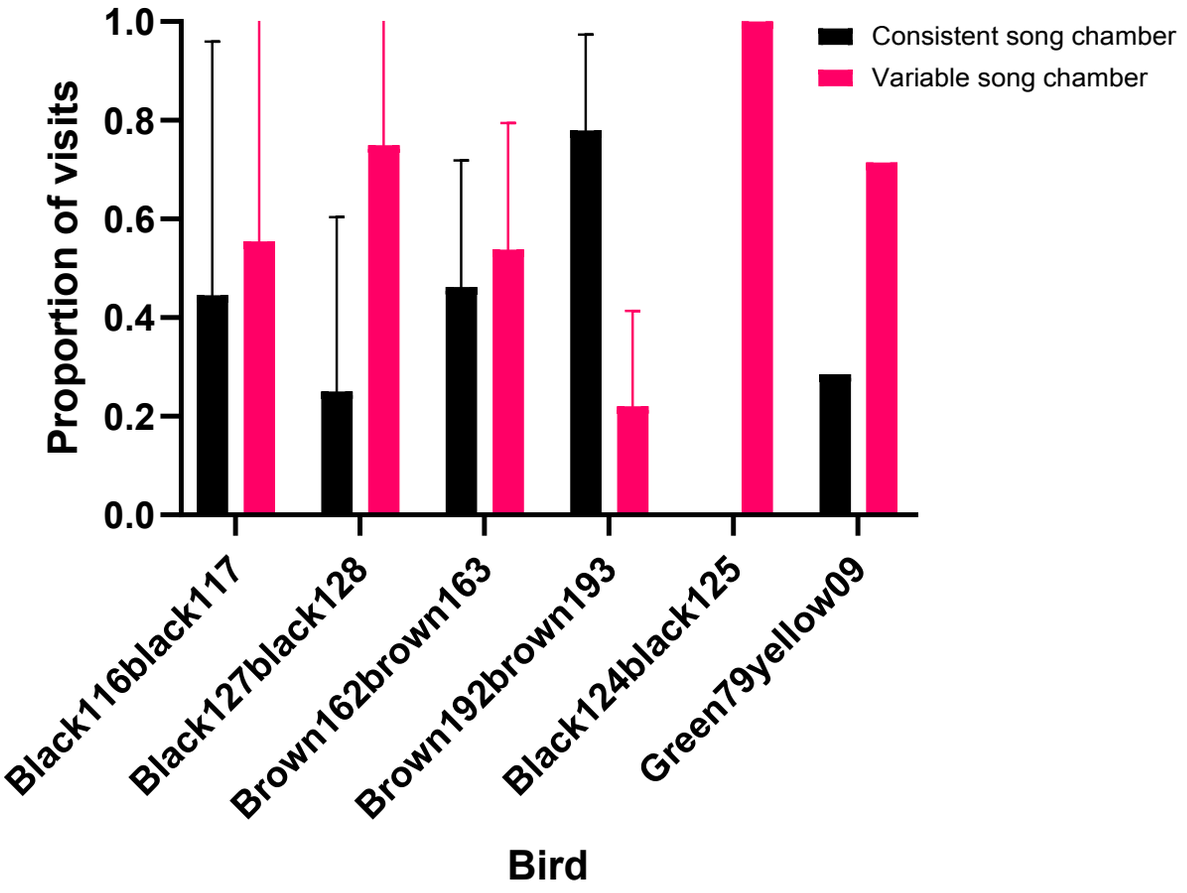



**Figure 17 Proportion of visits to consistent and variable song chambers**. Bars indicate the proportion of number of occurrences in the chambers near where consistent or variable songs were played. The error bars indicate the standard deviation.

Wilcoxon signed rank test on this data (p-value = 0.3125 ) indicated that there is no significant difference between the number of visits to the consistent song chamber and the number of visits to the variable song chamber. Thus, there is no significant preference for consistent song over variable song (or vice versa) when the number of visits is used as a measure for preference.

### 3.3.8 No significant preference using bird activity as a measure for preference

The activity levels of a bird during each stimulus were considered as another measure of preference, with the assumption that the birds are more active and switch chambers more frequently if they prefer one stimulus over another (Fig. 18). There was no significant difference between the two groups (Wilcoxon signed rank test; p-value = 0.4375 ).

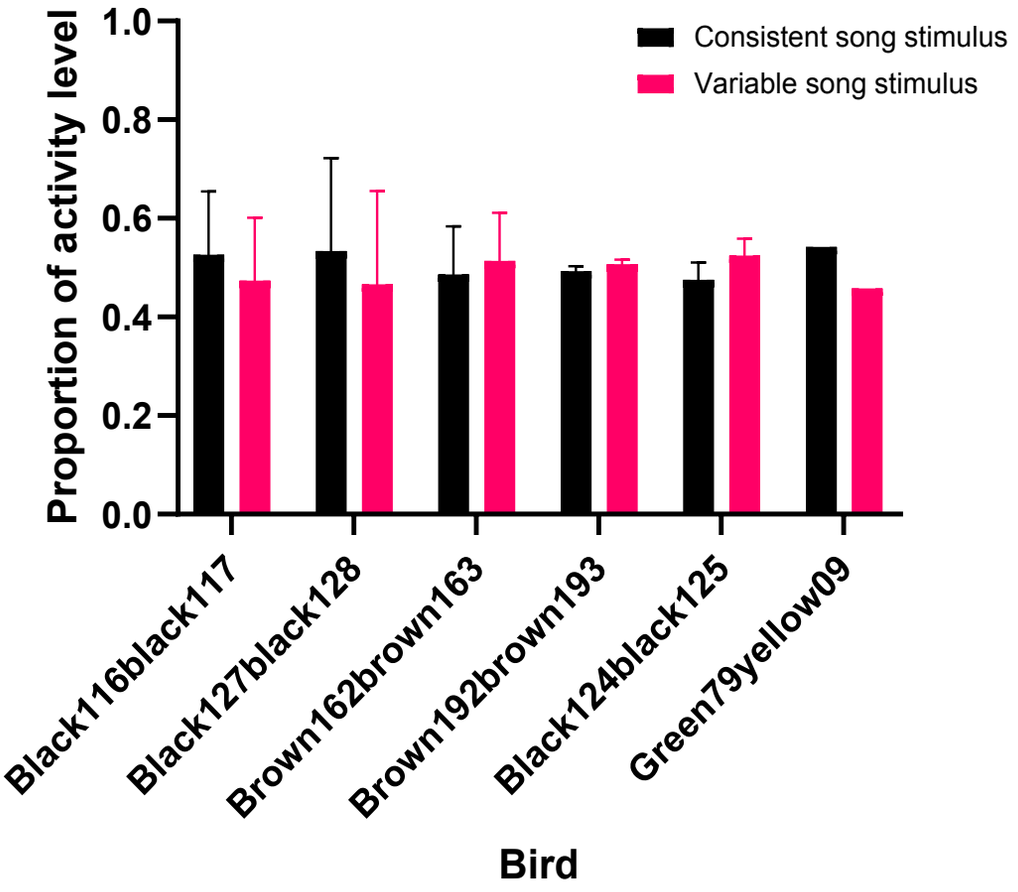



**Figure 18 Proportion of activity levels during consistent and variable song stimuli.** Bars indicate the proportion of each bird's activity level (measured by number of chamber transitions) when consistent song and variable song stimuli are played. Error bars indicate the standard deviation.

### 3.3.9 No significant preference using number of calls as a measure for preference

The number of calls given by the birds for each stimulus has been used as a measure for preference in previous studies. I calculated the proportion of calls given by the birds for each stimulus using the formulae listed in Table-3 (Fig. 19). A Wilcoxon signed rank test on this data (p-value > 0.99) showed no significant preference for consistent song over variable song.

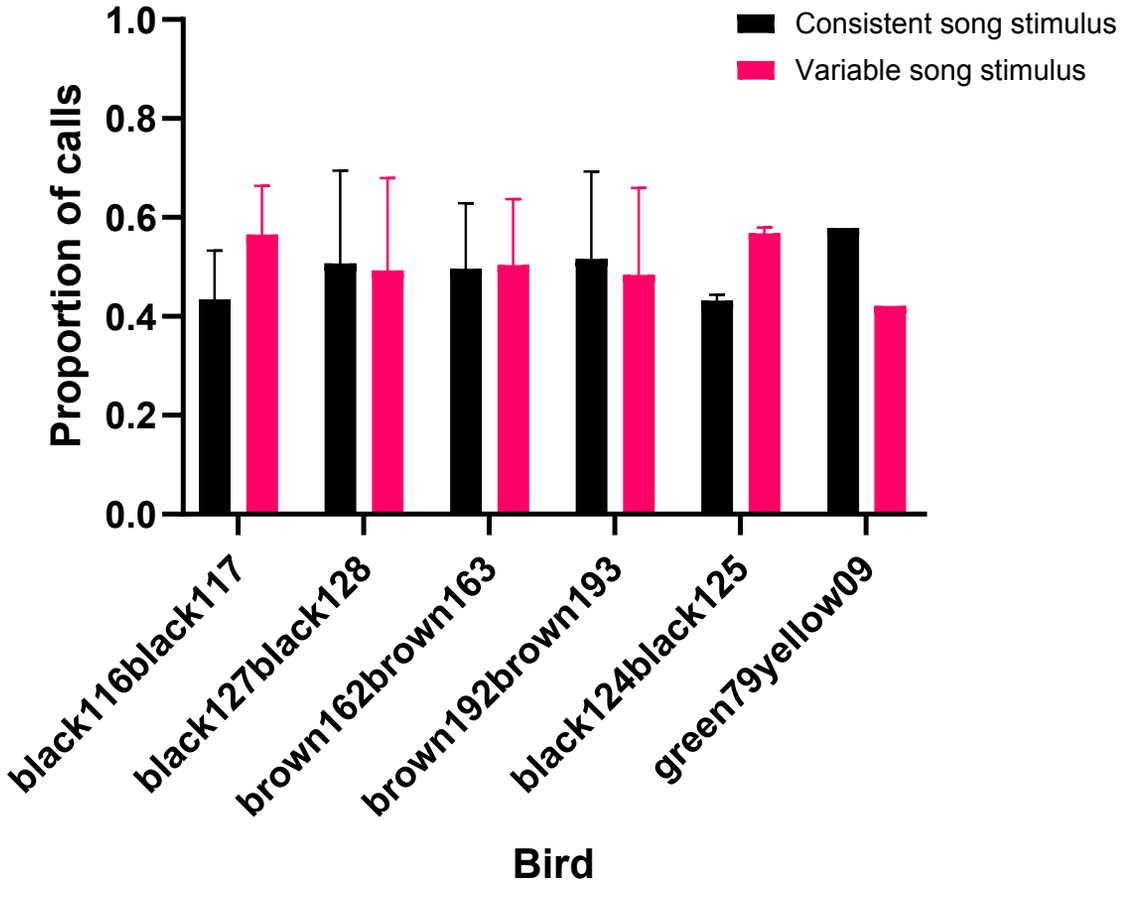



**Figure 19 Proportion of calls during consistent and variable song stimuli.** Bars indicate the proportion of number of calls given by the bird when consistent or variable song stimuli were played. Error bars indicate the standard deviation.

### 3.3.10 PI for duration and visits are correlated and PI for activity and calls are correlated

The Preference Index (PI) for each preference measure was calculated using the formulae listed in Table-3. The PI values for different preference measures of each experimental day of each bird were then correlated to check if the different

preference indices give similar information about the preference of the birds (Fig. 20). PI for duration and PI for number of visits are correlated (Spearman's r = 0.936), and PI for bird activity and PI for number of calls are correlated (Spearman's r = 0.721). The other preference measures are uncorrelated (Spearman's r < 0). The correlation between the PI for duration and PI for number of visits suggests that the birds spend more time in those chambers that they visit more often. Thus, in most of my data, there are only a low number of instances of the birds spending more time in a chamber due to inactivity. The correlation between the PI for bird activity and PI for number of calls suggests that when birds are more active and hop between chambers more often, they also give more calls.

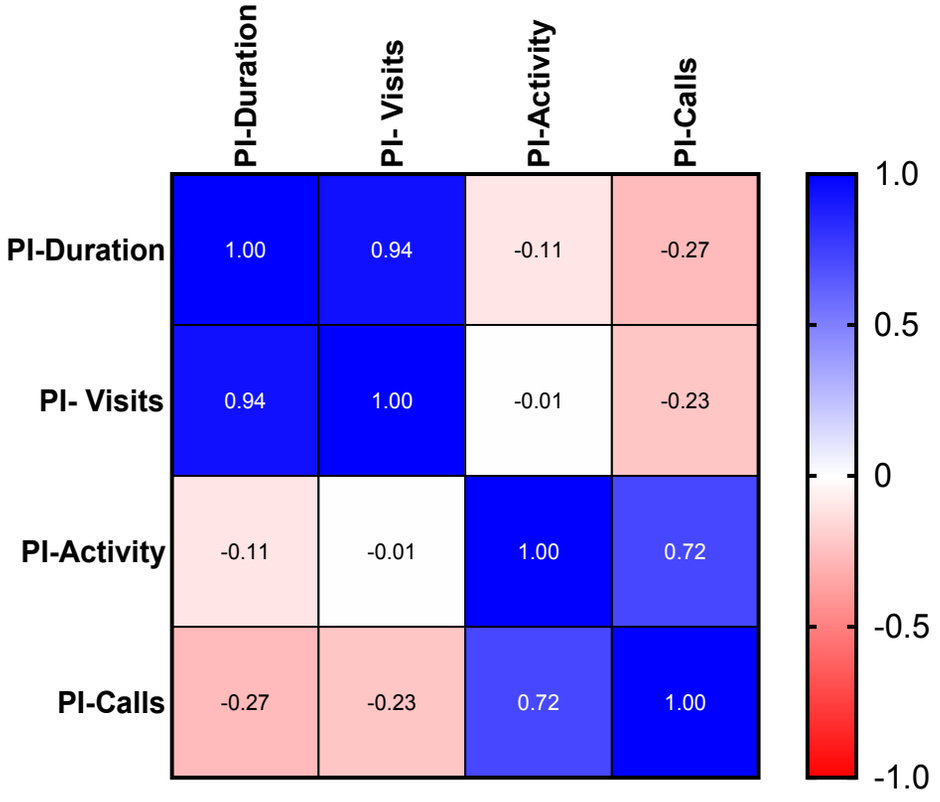



**Figure 20 Heatmap of Spearman's rank correlation of the preference indices.** PIs are measured using the duration spent in chambers, number of visits to each chamber, bird activity during each stimulus, and number of calls given by the bird during each stimulus

### 3.4 Dual choice classification task experiment

While preference assays measure natural preferences for specific stimuli, they do not tell us if the specific stimuli can be differentiated reliably, when associated with a reward. To test this, I trained two birds on a simple operant conditioning task where birds were expected to distinguish consistent and variable songs and indicate their

choice through hops onto specific perches. Birds were trained for 4-5 days each in Stage-1 of training. The first bird did not learn the task after the fourth day of training, and hence training was discontinued for that bird. The second bird has associated hopping on a perch with the food reward, and exhibited a lot of perch hops at certain times during the day. There were many technical problems with the construction of the setup, and also with training the birds (the birds would peck at the IR LEDs, eat the spilled seeds after many instances of the seed cup going back and forth in the cage, etc). However, currently the setup works and I am training the birds further through all the remaining stages.

# Discussion

The aim of this thesis was to determine if female zebra finches can perceive the syllable-level variability in the song of the male zebra finch, and prefer males which sing consistent songs. To test this, I designed two experiments which would provide complementary insights about the female's perception of variability in the zebra finch song. The first experiment was a phonotaxis-based preference assay, involving two stimuli being played on the two ends of a three-chamber cage. The aim of this experiment was to determine if the female zebra finches can naturally perceive variability in the song and exhibit a preference for consistent song over variable song. The second experiment was an operant conditioning dual-choice classification task experiment, in which the birds were trained to correctly classify a stimulus as consistent song or variable song. The aim of this experiment was to determine if the female zebra finches can distinguish consistent song and variable song after training. This experiment cannot inform us about whether the females can naturally distinguish consistent and variable song, but allows us to test whether females can distinguish them after training.

The results I got from the preference assay indicated that the birds have no preference for consistent song or variable song. However, these results do not provide any insights into whether the birds can distinguish between the two types of songs. Due to the nature of the operant conditioning task, I am yet to get results from the second experiment. If the birds are able to correctly classify the stimuli, it would suggest that they possess the capacity to discern the degree of variability in the song, but that it is not a relevant factor in their mate selection process. Conversely, if the birds are unable to distinguish between consistent and variable songs in the operant conditioning experiment, this would imply that birds lack the ability to assess the variability in song and that it holds no ecological significance to them. Prior to conducting the experiment, testing on the setup is required to ensure that it is fully operational. This would involve training and testing birds to perform a simpler task like distinguishing two different motifs.

Although many studies on mating preferences have used phonotaxis-based preference assays (Miller, 1979; Clayton, 1988), it is not clear if approaching a stimulus indicates sexual preferences or just a preference for association in a non-sexual context. Thus, positive results from such preference assays could indicate

that the approach is affiliative, but does not inform us about the mating preferences of the birds (Searcy, 1992). In my study, in order to analyse the preference of the birds, I used four measures: Time spent by the birds in each of the side chambers, the number of visits to each of the side chambers, the activity of the birds during a stimulus, and the number of calls given by the birds during a stimulus. The preference indices for the time spent in a chamber and the number of visits to a chamber were found to be correlated, while the preference indices for bird activity and number of calls are correlated. However, the preference index for the time spent in a chamber and the number of calls are not correlated. Since these two measures have been to quantify the preference of female zebra finches (Miller, 1979; Clayton, 1988, Chen *et al*., 2017), it would be interesting to see if they are correlated for other preference experiments in which the birds show a preference using one of the measures.

The caveats of my study are that the sample size is low, and the conditions for each bird were slightly different. My sample size comprises of 6 birds, out of a total of 21 birds that I conducted the preference assay on. This showed that only ~30% of birds used, responded during the preference assay. Through the course of my experiments, I have tested different possible ways to increase the response from the birds, that I outline here. After the primary set of experiments in which the birds were not responsive, I decided to train the birds before doing the experiment on them. This slightly improved the response of the birds, and helped to identify potentially responsive birds for the experiment. While pre-training through exposure to live male birds helped identify potentially responsive birds, it did not increase the number of responsive birds. Second, for the first two batches of birds, both the control and test conditions were done in the preference assay. However, due to the potential for unintended associations between the birds and the sides due to the control stimuli, the experiment, the experiment was subsequently modified to include only the test stimuli. Given that the subsequent experiments lacked control conditions, it became necessary to carry out a set of experiments in the same birds by swapping the sides of the consistent and variable song stimuli in order to eliminate inherent side-preference biases. Changing the stimuli to include only the test conditions did not affect the responsiveness of the birds, but facilitated the interpretability of the results.

Third, I tested whether the directed songs (songs sung towards a female) were more potent stimuli for preference assays. I tried using stimuli constructed from directed

song instead of undirected song for one set of experiments. This modification did not increase the responsiveness of the birds either. Thus, using undirected songs as stimuli was not the reason for the non-responsiveness of the birds. Fourth, I used female birds which were pair-bonded to male-birds by using the undirected song playback of the pair-bonded male in the experiment. This method, which had been previously used in other such preference assays (Miller, 1979; Woolley and Doupe, 2008), did not yield the expected levels of responsiveness. Thus, in an attempt to increase the sample size, each bird underwent slightly different conditions. Overall, these results show that pre-training through exposure to live males, using directed songs, and using the song of the pair-bonded male bird, did not motivate the birds to switch chambers.

There are other methods to test the preference of the birds, and these methods may vary in practicality, sensitivity and interpretability (Searcy, 1992). One such method is using operant conditioning to train the birds to peck two keys corresponding to two different stimuli, and later measuring the number of times they peck each key as a measure of preference (Houx and Ten Cate, 1999; Riebel, 2000; Riebel *et al.*, 2002; Riebel and Smallegange, 2003). Measuring the copulation solicitation displays (CSD) of the birds is a widely used method to study the mating preferences of the birds. In many such studies, oestradiol is delivered to the birds to increase the CSD of the birds (Moore, 1982, 1983). Other studies that used a phonotaxis-based preference assay have used a sample size of 20 birds (Miller, 1979; Woolley and Doupe, 2008). In one such study, they used female birds which were pair-bonded with their mates for a period of six months to two years (Miller, 1979). In another study, the birds were pair-bonded for at least three months, and in some cases up to three years (Woolley and Doupe, 2008). In my study, I paired the birds for just 7 to 10 days before isolating them as I had practical constraints in doing the experiment. Thus, for future preference assay experiments, keeping the birds pair-bonded for a duration of at least three to four months might yield a bigger sample size.

In conclusion, the results from my study seem to suggest that female zebra finches do not exhibit a preference for consistent songs over variable songs. This indicates that song consistency, at the level of syllable features, may not be relevant in the context of mate choice by the female zebra finch. However, the results from a previous study (Woolley and Doupe, 2008) suggest that female zebra finches do exhibit a preference for songs which are consistent in terms of the fundamental

frequency of stacked syllables in the motif. This result was from a correlation, and was not directly tested. Thus, to proceed with this project, artificial stimuli with motifs varying at the level of only the fundamental frequency of stacked syllables can be used in the preference assay. In order to get more conclusive results, future directions would include doing other preference assays such as the operant conditioning key-peck assay described earlier (Houx and Ten Cate, 1999; Riebel, 2000; Riebel *et al.*, 2002; Riebel and Smallegange, 2003). Also, the results from the dual choice classification task experiment would complement the results from the preference assay and thus reveal more information about the ability of the birds to distinguish variable song from consistent song.

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