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# Relative Salience of Syllable Structure and Syllable Order in Zebra Finch Song

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## Abstract

There is a rich history of behavioral and neurobiological research focused on the 'syntax' of birdsong as a model for human language and complex auditory perception. Zebra finches are one of the most widely studied songbird species in this area of investigation. As they produce song syllables in a fixed sequence, it is reasonable to assume that adult zebra finches are also sensitive to the order of syllables within their song; however, results from electrophysiological and behavioral studies provide somewhat mixed evidence on exactly how sensitive zebra finches are to syllable order as compared, say, to syllable structure. Here we investigate how well adult zebra finches can discriminate changes in syllable order relative to changes in syllable structure in their natural song motifs. And we identify a possible role for experience in enhancing sensitivity to syllable order. We found that both male and female adult zebra finches are surprisingly poor at discriminating changes to the order of syllables within their species-specific song motifs, but extraordinarily good at discriminating changes to syllable structure (i.e. reversals) in specific syllables. Direct experience or familiarity with a song, either using the bird's own song (BOS) or the song of a flock mate as the test stimulus, improved both male and female zebra finches' sensitivity to syllable order. However, even with experience, birds remained much more sensitive to structural changes in syllables. These results help to clarify some of the ambiguities from the literature on the discriminability of changes in syllable order in zebra finches, provide potential insight on the ethological significance of zebra finch song features, and suggest new avenues of investigation in using zebra finches as animal models for sequential sound processing.

### Keywords

auditory perception; hearing; song motif; syllable sequence; syllable structure

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**Ethics Statement**
This work was conducted in accordance with Association for the Study of Animal Behaviour (ASAB) guidelines and was approved by the Institutional Animal Care and Use Committee (IACUC) (R-15-09), University of Maryland, College Park.

# Introduction

Songbirds have been key model organisms for neurobiological studies of vocal learning and production (Fee and Scharf 2010; Brainard and Doupe 2013). The relation between production and perception is becoming an increasingly important question within the field of behavioral neurobiology and animal cognition; however, compared to what is known about vocal production, much less is understood about species-specific vocal perception across songbirds (see, for example, Ball and Hulse 1998).

Among songbirds, zebra finches (*Taeniopygia guttata)* are one of the most widely studied species for both behavioral and neuroscience studies investigating song production (Fee and Scharf 2010; Griffith and Buchanan 2010). Male zebra finches each learn one song from a tutor which is comprised of a varying number of introductory notes or calls, followed by a repeated group of 3–8 syllables (a single harmonic vocalization defined by the time waveform), and a stereotyped sequence of these song syllables comprising a motif (Immelmann 1969; Arnold 1975; Zann 1996). By 90 days post-hatch, zebra finch song is crystallized, meaning that both the sequential structure of the motif and the acoustic structure of the individual syllables remain highly consistent (Immelman 1969; Menyhart et al. 2015). Once a male reaches adulthood, there is generally more acoustic variation between individual syllables than there is in the order of syllables within a motif (e.g. Zann 1996; Sturdy et al. 2000). Despite the inflexibility of syllable order in adult song, it would make sense that zebra finches would be sensitive to both syllable structure and syllable order because they successfully copy these features during the critical period for song learning and maintain them throughout life.

As important as the perceptual component of vocal learning is to zebra finches, surprisingly few behavioral studies have specifically tested the sensitivity of zebra finches to changes in syllable order within a natural song motif. One exception is a study by Braaten et al. (2006) where male and female adult and juvenile zebra finches were trained with operant conditioning on a Go/No-Go classification task to “Go” to a normal motif and to not respond (i.e. “No-Go”) to a completely reversed motif where both the syllables and the syllable order were reversed. Once this classification task was learned, the birds were then tested on a set of stimuli that included the original training motifs and two probe stimuli. One of these probe stimuli reversed each syllable in the motif keeping their order intact and the other probe stimulus used the normal forward syllables but reversed the order of their occurrence in the motif. The birds responded to the probe motifs with reversed syllables as they did to the entire reversed motif, indicating that they discriminated forward from reversed syllables. Surprisingly, they responded to motifs with syllables out of order more like they did to a normal motif with syllables in their natural order, indicating they heard little difference between the two motifs. With repeated training (1800–4800 trials) and punishment during training for responses to the order reversed motif in addition to the completely reversed motif, juvenile finches were eventually able to respond at the level of 75% correct to forward motifs and withhold a response to order-reversed motifs. In a similar study, Bengalese finches (*Lonchura striata domestica*), a species with a more flexible syllable order, also showed only weak ability to respond differently to motifs that involved a change in syllable order (Okanoya et al. 2000).

Beyond these two studies with normal and altered motifs, there have been a number of behavioral studies focused on the perception of artificial grammar - examining the birds' ability to use rules about sequence to discriminate short strings of song elements. Broadly speaking, these studies show that zebra finches can classify short strings that differ in the order of elements but it is not always clear the extent to which they rely on non-sequential features such as the identity of the final or first syllable or the presence of repeated syllables (Chen et al. 2015; van Heijningen et al. 2013; van Heijningen et al. 2009). It is specifically not clear whether birds are responding to the location of a particular syllable or syllables or to a global characteristic of the entire sequence (Beckers et al. 2017). Regardless, these studies all point to the challenge in determining exactly how sensitive adult zebra finches are to syllable order even though it is abundantly clear that young males, at least, clearly learn such order from external models at an early age (see, for example, Menyhart et al. 2015; Baran et al. 2017; Lipkind et al. 2017).

While there are relatively few studies on the perceptual consequences of syllable order in natural motifs, there are a number of studies showing that zebra finches are exquisitely sensitive to the acoustic fine structure of natural calls and song syllables (Dooling et al. 2002; Lohr et al. 2006; Vernaleo and Dooling 2011; Dooling and Prior 2017). Though it is unrealistic to think that the perceptual behavior of the whole organism can be fully reflected in electrophysiological recordings from discrete nuclei in avian auditory and song circuits, the physiological data from various recording sites are in rough agreement with these behavioral studies pointing to a primacy of syllable structure over syllable order (see, for example, Vicario and Yohay 1993; Doupe 1997; Miller-Sims and Bottjer 2014).

What accounts for this large behavioral difference in the perceptual sensitivity of zebra finches to syllable structure versus syllable order in natural song motifs when both song characteristics are clearly learned during the critical period? One possibility is that different behavioral tasks were used to measure sensitivity to syllable order versus syllable structure. The Go/NoGo task used by Braaten et al. (2006) to test the birds' ability to hear syllable order in a natural motif is a classification task. Such tasks involve some long-term memory processes (i.e. the bird must hold a template for the 'Go' stimulus and the 'NoGo' stimulus in long term memory because the trial-to-trial interval between sound presentations in such a classification task is often on the order of tens of seconds). By contrast, the 'Go/NoGo' task with a repeating standard or background - as used here to test the zebra finch's ability to detect differences in the temporal fine structure of syllables (e.g. Vernaleo and Dooling 2011) - is more properly called a discrimination task. In such a task, there is little memory load and no need to learn an acoustic category. That is, the bird is not trained to respond to a particular stimulus or class of stimuli, but rather to respond to a difference between two stimuli that occur in quick succession usually separated by only a few hundred milliseconds (e.g. Dooling and Okanoya 1995).

The previous psychophysical methods showing that zebra finches are exquisitely sensitive to syllable structure (for review, Dooling and Prior 2017) have not yet been used to assess sensitivity to syllable sequence, which is the main goal of the present experiments. Using natural song motifs as the standard, we compared the discriminability of changes in syllable structure to the discriminability of changes in syllable order in natural motifs using the exact same discrimination task and reinforcement contingencies for both types of stimuli. An assortment of changes in syllables and syllable sequences was used to provide a broad sense of which of the two classes of changes is more salient. Additionally, to provide a broader comparative framework for understanding the data on zebra finches, we also tested three canaries (*Serinus canaria*) (a songbird) and two budgerigars (*Melopsittacus undulates)* (a parrot), both of whom exhibit vocal learning.

## General methods

### Subjects

The subjects in these experiments were adult male and female zebra finches, canaries and budgerigars. The age of the subjects ranged from 8 to 16 months at the start of the experiment. Birds were housed individually in close proximity to all other birds in a mixed vivarium of zebra finches, canaries, and budgerigars. Though physically separated, subjects remained in visual and auditory contact with birds in the vivarium throughout the duration of these studies. The subjects were kept on a light cycle of 8L:16D throughout the duration of the experiment. Birds were mildly food deprived to provide motivation and their diet was carefully controlled such that they were maintained at about 90–95% of their free feeding weight. White hulled millet was used as a food reward in the testing apparatus and birds received an additional portion of pellet or mixed seed at the end of the day. Birds also had access to grit, and, occasionally, vegetables, fruit, or hard-boiled egg.

### Apparatus

Birds were tested in an operant conditioning chamber previously described elsewhere (Dooling and Okanoya 1995) and routinely used with finches (Vernaleo and Dooling 2011). Briefly, the apparatus consisted of a small wired cage (23x25x16cm) in an IAC-3 sound isolation chamber (Industrial Acoustics Company, Inc., Bronx, NY). A custom-made response panel with two microswitches was attached to the cage wall. Attached to both microswitches was a light-emitting diode (LED). The left LED served as an observation key, and the right LED served as a report key. Custom software written in MATLAB (Mathworks, Natick, MA) was used to program Intel Core 2 Duo computers that ran Tucker-Davis hardware. This program controlled input to the apparatus through a Tucker-Davis Technologies System 3 DD1 stereo analog interface (Tucker-Davis Technologies, Alachua, FL). The stimuli were then sent through a Crown D-75 amplifier (Crown International, Inc., Elkhart, IN), and finally to an Orb full range point source speaker (Model Mod1) which was placed 40 cm above the bird’s head when standing on the perch. An opening at the foot of the perch with an automated hopper allowed for reinforcement with food during testing. The amplitude of stimuli was adjusted such that they played at ~65dBA in the apparatus. Sound level was measured in the free field with the ” microphone of a BK precision sound level meter (Model #732) placed 7 cm above the bottom of the cage, at approximately the location of the bird’s head when it was positioned in front of the observation key.

### Stimuli

**Recording—**Stimuli consisted of natural zebra finch song motifs which were either familiar (BOS or colony mate) or unfamiliar (novel) to the birds running on the discrimination task. Unfamiliar song motifs were recorded in 2010 (Vernaleo and Dooling 2011) from birds that were no longer present in the colony and whose vocalizations were never heard by birds in this study. These unfamiliar stimuli were recorded individually from 4 male zebra finches housed individually in a foam-lined acoustic chamber using a Marantz portable solid-state recorder (Model PMD670) at a sampling rate of 48 kHz.

Additionally, we recorded song from zebra finches used in the current study to use as familiar or BOS stimuli. These songs were recorded in a foam-covered room by tie-clip microphones (AKG C417) and a Zoom F8 multitrack field recorder at a sampling rate of 44.1 kHz. All of these recordings were resampled at 24,414Hz for playback in the psychoacoustic set up using TDT equipment (described above).

**Preparation of Natural Motifs—**All songs were further processed in the following way: Using Adobe Audition (ver: 2015.2) one representative motif from each male’s song was extracted, and high-pass filtered with a cutoff frequency of 350 Hz. The intervals between syllables were band-reject filtered at all frequencies such that there was silence between syllables. Lastly, a 5-ms cosine rise and fall time was placed on all 4 motifs to ensure smooth onsets and offsets. This did not affect the overall envelope of the syllables within the motifs. In all, motifs ranged in duration from 650–905ms, and contained 5–7 syllables.

**Reversed Syllable Motifs—**Four different syllable reversal modifications and three different syllable shuffle modifications to each of the four natural motifs were created. Examples of a natural motif and three targets (two syllable reversals and one shuffle) are shown in Figure 1. Syllable reversal modifications consisted of a reversed version of the entire motif, a syllable-reversed motif, and two versions of the motif where a single syllable was reversed. Reversed targets were created from the natural motifs using the “reverse” tool in Adobe Audition. Syllable-reversed targets were created by selecting all syllables separately and reversing them in place, so that the order was maintained but the spectro-temporal structure of each syllable was altered. Two versions of a single syllable-reversed target were created: one with the second syllable reversed, and one with the second to last syllable reversed. While the differences between forward and reversed stimuli are readily apparent in these spectrograms, they are not easily distinguished by humans listening to these motifs (e.g. Lohr et al. 2006).

**Shuffled Syllable Motifs—**The three different sets of shuffled syllable motif consisted of a fully shuffled motif where syllables were played in random order, a motif where the first syllable remained in position, but the rest were randomly shuffled, and a motif where the last syllable was moved to the first position and the rest of the motif remained unchanged. One example of a shuffled motif is shown in Figure 1. Reordering of syllables was done in Adobe Audition by selecting each syllable and creating a new sound file where the syllables were placed in new positions according to a random number generator. Inter-syllable intervals were kept the same as in the original motif. In contrast to the case with reversed syllables, a change in the order of syllables in a motif is generally distinguishable for the casual human listener.

## Training and Testing Procedure

The training and testing procedures have been fully described earlier (e.g. Vernaleo and Dooling 2011; Supplemental Figure 1). Training proceeded in several stages. The birds were first trained in two daily, 30–45 min sessions for several days to peck an observation LED, which triggered a tone and a food reward. Once this was learned, the birds were moved through several additional stages of training each lasting a few days. The sequence of events during a trial consisted of several distinct phases. In the initial phase, a peck on the observation key initiates an observation phase and begins a random time interval of 2–7 sec. Following this variable interval, a second peck on the observation key initiates the report phase of the trial in which the target stimulus alternates with the background stimulus. The birds first learn to peck the observation key while a tone of one frequency is repeated at the rate of 2/sec (i.e. the background) and to peck the response key when they detected a change (e.g. a target tone of a different frequency). After a few days (i.e. a few hundred trials), song motifs were substituted for tones. Within a few trials, the birds would learn to peck the observation key during the repeated presentation of a background motif and to peck the report key only when a new motif (i.e. the target) was alternated with the background motif. To ensure that birds generalized, different motifs and motif modifications were used for each bird in this phase of training. Birds received a 2-sec access to food if they pecked the report key within 3–4 sec (i.e. response interval) of the onset of the target motif. Generally, this training phase, moving from a completely naïve bird to a fully trained bird, lasted about 2 weeks.

In these and following experiments, the natural motifs used during testing were of slightly different durations (sometimes with different numbers of syllables) but these motifs were presented at a such a rate (usually 1/sec) that there was always approximately a 300 ms interval between the end of one motif and the beginning of another. The response interval (i.e. between about 3–4 s) was adjusted accordingly depending on the motif length so that two presentations of the target motif (separated by one presentation of the background motif) could occur within the response window. Each test session lasted about 20–30 minutes and consisted of 100 trials occurring in 10 blocks of 10 trials each. In the following experiments, each block of ten trials contained all seven modified motif targets (four motifs with reversed syllables, three motifs with shuffled syllables) and three sham trials (i.e. presentation of the background motif as the target) all presented randomly.

Birds were run daily, usually in two 100-trial sessions, until there were at least 250–300 consecutive trials collected on a given motif in which there was no further improvement in the hit rate or false alarm rate. The average false alarm rate in these last few hundred trials across all birds was about 10% over all of these experiments. Thirty percent of the trials in a 10-trial block were sham trials in which no target was presented. False alarm rates were calculated from the number of responses to sham trials. If the bird pecks the report key during a sham trial, the lights in the test chamber are extinguished for a period of 3–10 seconds, depending on the bird's propensity for false responding. The duration of the lights off punishment period was adjusted periodically to maintain the false alarm rate at about 10% across sessions. Birds were tested, using the above positive reinforcement and punishment contingencies, over a period of many months on a near daily basis on various versions of natural and modified zebra finch song motifs where both reversed syllable and shuffled syllable motifs occurred in every 10-trial block. Past research has shown these methods minimize or eliminate the possibility that early training influences alter perceptual performance or that birds may selectively respond to some discriminable contrasts but not to others (Klump et al. 1995).

Finally, in addition to hit rate and false alarm rate, response latencies were also measured on every trial. Response latencies were defined as the time elapsed from the onset of the target motif until the bird responded by pecking the report key.

### Analysis

We analyzed birds' performance in two ways. A bird's behavior (hit/miss/correct) and rejection/false alarm) on each trial was recorded and later pooled together to calculate an averaged hit rate and false alarm rate (Tu and Dooling 2012, 2017). Then, to minimize any species differences in response bias, these two numbers were used to derive a d′:

$$d' = z(Hit\ rate) - z(False\ alarm\ rate)$$

To avoid infinite values, 100% correct and 0% false alarm rates were converted to 1/(2N) and 1 − 1/(2N), respectively, where N is the number of trials on which the percentage was based (Macmillan and Creelman 2005). Evaluating differences in d′ between two conditions was done in the conventional way. The standard error (square root of the variance) of d' (Macmillan and Creelman 2005; Gourevitch and Galanter 1967) was calculated.

Secondly, we compared birds' performance using d' (and latency values, see supplemental information) on the targets for each experiment using inferential statistics. All statistical analyses were carried out in R (v.3.2.3, R Foundation for Statistical computing). We used linear-mixed models (function lmer from the lme4 Package). Tukey's follow up tests were conducted on significant main effects. Prior to interpretation, we checked the validity of each model by plotting the distribution of the residuals.

## Experiment 1–Discrimination of Syllable Structure and Syllable Order in Unfamiliar Zebra Finch Song Motifs

In this experiment, birds were tested on their ability to discriminate between natural, unfamiliar song motifs and versions of these same motifs that were altered by either reversing all or some of the syllables or shuffling the order of syllables. The motifs used in this study were unfamiliar to the current study birds because they were recorded from birds used in a previous study to whom the birds in the current study had never been exposed (Vernaleo and Dooling 2011). The main test subjects here were 7 adult zebra finches (4 male, and 3 female). To determine whether our results were unique to zebra finches, we also tested three canaries (a songbird) and two budgerigars (a parrot) on the exact same set of stimuli. In sum, 7 adult zebra finches (4 male, 3 female), 3 adult canaries (2 male, 1 female), and 2 adult budgerigars (1 male, 1 female) were all tested on the exact same set of unfamiliar zebra finch motifs and their respective modifications. The order of testing different motifs was randomized across all birds.

## Results and discussion

Zebra finches performed very well on each of the four modified motifs involving reversed syllables and significantly worse (i.e. lower d') on modified motifs involving shuffled syllables (Figure 2, A) (Stimulus, $\chi^2$=983.15, DF=6, P<0.001; Tukey's HSD; Reversals vs Shuffles all P<0.001). On these three shuffled targets, zebra finches also performed significantly worse when the first syllable remained in its position (Tukey's HSD; P<0.001). There were no differences between male and female zebra finches (Sex, $\chi^2$=0.08, DF=1, P=0.784; Sex*Stimulus Target $\chi^2$=6.78, DF=6, P=0.342).

Our finding that zebra finches were able to easily discriminate the changes in syllable structure that occurred by reversing the syllable is consistent with previous research showing zebra finches can detect subtle changes to temporal fine structure in their vocalizations and in synthetic stimuli such as Schroeder harmonic complexes (Dooling et al. 2002; Lohr et al. 2006; Vernaleo and Dooling 2011). Zebra finches were much less sensitive to changes in the sequence of syllables in shuffled motifs, which mirrors results from the study by Braaten et al. (2006) who reported that both adult and juvenile zebra finches responded to order-reversed motifs much as they did to natural, forward motifs in a classification task. Also, our zebra finches performed worst when the first syllable remained in place, which is consistent with evidence from other studies that different syllable locations within a motif may have different salience for birds categorizing altered motifs (e.g. van Heijningen et al. 2013; Chen et al. 2015; Chen et al. 2016; Beckers et al. 2017).

Comparisons of zebra finches (Figure 2A) with canaries (Figure 2B) and budgerigars (Figure 2C) revealed that the three species performed differently on reversed and shuffled targets (Canaries Stimulus, $\chi^2$=202.81, DF=6, P<0.001; Budgerigar Stimulus, $\chi^2$=26.86, DF=6, P<0.001). Canaries performed similarly to zebra finches in that they were much better on discriminating reversed than on shuffled targets (Tukey's HSD; Reversed vs Shuffled all P<0.001; Figure 2B). Canaries also performed better when every syllable in the motif was reversed (P<0.002). By contrast, budgerigars performed nearly as well on both the reversed and shuffled targets, but they performed worse on the shuffled target where the first syllable remained in its original position (all P<0.005; Figure 2C).

Mean response latencies and standard errors for each species and each stimulus contrast are given in Supplemental Information (Supplementary Figure 2). For all three species, the birds showed the shortest response latencies to the whole song reversal and all syllable reversals (where each syllable is reversed in place) and showed longer response latencies to target motifs involving single syllable reversals or shuffled syllable. This pattern of response latencies also shows that discrimination of syllable reversals occurring later in the target motif, as well as the syllable shuffled target motifs, are more difficult to discriminate than target motifs in which all syllables were reversed.

## Experiment 2–Discrimination of Syllable Structure and Syllable Order in Familiar Zebra Finch Song Motifs - A Possible Role of Experience

Studies of song learning focus almost exclusively on the brain and behavior of males throughout development as they learn to produce their song by hearing a model. Indeed, there has been extensive electrophysiological research focused on the neural mechanisms underlying the perception, production, and learning of the BOS in male zebra finches (Doupe 1997; Lewicki 1996; Vallentin and Long 2015; Danish et al. 2017). There is, of course, plenty of evidence that experience affects perception more broadly. Social experience can be essential in enhancing song learning in males (Chen et al. 2016; Baran et al. 2017; Beecher 2017). Acoustic and social experiences during song development have profound effects on song production in adult male songbirds (for reviews, see Konishi 1985; MacDougall-Shackleton 2009). And female zebra finches, though they do not produce song, nevertheless show evidence of learning, memorizing, and categorizing aspects of song that they have heard (Kriengwatana et al. 2016; Hauber et al. 2013; Holveck and Riebel 2014).

### Additional Methods

The methods used to test changes in syllable order versus syllable structure were the same as Experiment 1. Here we compared the performance of four males on unfamiliar song motifs (Experiment 1), their own song motifs (i.e. BOS), and familiar motifs selected from male birds housed in the same large aviary as the test birds. A motif extracted from each male’s song was used as the natural motif background stimulus. As in Experiment 1, the seven altered motifs served as the targets to be discriminated from the natural motif which was the repeating background. Three female finches tested on familiar and unfamiliar motifs performed similarly to males (see Supplemental Information).

### Results and discussion

Familiarity improved performance (Males, Stimulus, $\chi^2$=435.26, DF=6, P<0.001; Familiarity, $\chi^2$=29.41, DF=1, P<0.001; Familiarity*Stimulus $\chi^2$=41.73, DF=6, P<0.001). Female finches performed similarly (see supplemental results). The results for the four males tested are shown in Figure 3. Familiarity, either from hearing the test motif in the home aviary or because the motif was the BOS, influenced the males’ performance on this task (Tukey’s HSD, Familiar-Unfamiliar, P=0.030; BOS-Unfamiliar, P=0.048). While familiarity did not affect the high level of performance on the reversed targets, it did considerably improve the birds’ performance on each of the shuffled syllable motifs. This is particularly apparent for the First Same Shuffled target on which birds performed particularly poorly on unfamiliar motifs. Interestingly, male birds did not perform better on manipulations of their own motif (BOS) relative to manipulations of other familiar motifs (Tukey’s HSD, BOS-Familiar, P=0.816).

## Experiment 3 - Discrimination of Syllable Structure and Syllable Order in Synthetic Zebra Finch Song Motifs

The previous experiments show that zebra finches are relatively insensitive to syllable sequence compared to syllable structure in their natural song motifs. In a natural motif, each syllable is acoustically distinct from every other syllable in the motif. These differences are highly salient to zebra finches and one question that arises is whether simplifying the acoustic differences between syllables might reduce the performance on syllable reversals, or conversely, whether it might enhance the performance on syllable shuffles. Here, we used MATLAB to generate test stimuli made up **of** Schroeder waveforms that matched the fundamental and harmonics of zebra finch vocalizations. Positive and Negative Schroeder waveforms are synthetic harmonic complexes that eliminate any differences in overall envelope and spectrum but differ on whether frequency sweeps up or down within a period–i.e. the fine structure. Constructing a synthetic motif of these waveforms means that the only cue available to the bird in discriminating syllable reversals is the fine structure, and the only cue for discriminating syllable order is tempo, essentially the amplitude envelope of the whole motif. Early work showed that zebra finches can discriminate a single reversed synthetic Schroeder syllable placed in a zebra finch song motif (Vernaleo and Dooling 2011). In this experiment, each syllable in the synthetic motif has exactly the same spectrum, amplitude, and temporal fine structure.

### Additional Methods

The subjects for this experiment were three zebra finches (1 male, 2 female), all used in Experiments 1 and 2.

**Background and target stimuli—**One natural motif from Experiment 1 served as model for creating a synthetic motif made of a Schroeder harmonic complex. This synthetic motif served as the repeating background stimulus in this experiment. As in the earlier study (Vernaleo and Dooling 2011), the Schroeder waveforms used to create the syllables all had a fundamental frequency of 640 Hz, which is about the average fundamental frequency for both zebra finch contact calls (Simpson and Vicario 1990) and zebra finch song syllables (Williams et al. 1989; Williams 2001). These waveforms consisted of ten harmonic components resulting in a frequency range of 640–6400 Hz with a 10 ms rise/fall time. Schroeder waveform “syllables” and intervals were the same duration as natural song syllables and intervals from a motif used in Experiment 1.

The Schroeder syllables were concatenated with intervals equal to those in the natural motifs so that the resulting stimulus was a string of Schroeder waveforms making up a Schroeder motif. A spectrogram, time waveform, and an example of several periods of the time waveform are shown in Figure 4. For the background Schroeder motif, only positive Schroeder waveforms were concatenated. For the target motifs, just as the experiments with natural motifs, either the entire motif was reversed, all the syllables were reversed in place (i.e. negative Schroeder waveforms), or single syllables were reversed just as in Experiment 1. Shuffled motifs followed those of Experiment 1 as well. In all, we ran 3 zebra finches on these stimuli and then we further tested 2 finches on a stimulus set in which the synthetic syllables were kept in the correct order, but the intervals were shuffled.

### Results and discussion

Zebra finches discriminated reversed synthetic syllables as well as they discriminated reversed natural syllables (Figure 5A). Additionally, as with the natural motifs, zebra finches performed much worse on shuffled synthetic syllables than reversed (Stimulus, $\chi^2$=199.69, DF=6, P<0.001; Tukey's HSD reversals vs shuffles all P<0.001). Zebra finches performed equally poorly on all of the shuffled targets for the synthetic motif.

In all three experiments, when syllables were shuffled in the natural or synthetic motifs, the intervals in between syllables were kept in their natural order and only the syllables were shuffled. So, as an additional test, we prepared a synthetic motif which shuffled the intervals, as opposed to the syllables. In other words, we kept the Schroeder syllables in the correct order and shuffled only the intervals. This altered the temporal envelope, or “tempo”, of the whole motif without changing syllable structure or syllable order. The two finches that ran on this test set were as insensitive to the shuffling of intervals in the synthetic motif as they were to the shuffling of synthetic syllables (Figure 5B).

## General Discussion

Zebra finches are closed-ended vocal learning birds that learn one song early in life which remains fixed for adult life (e.g. Zann 1996). Since juvenile males learn both syllable structure and syllable order early in life, it is somewhat puzzling that, as adults, these birds easily discriminate reversed syllables but are not particularly sensitive to changes in the order of syllables in unfamiliar zebra finch song motifs. This asymmetry in performance is all the more intriguing because changes in syllable order, but not syllable reversals, are fairly easy for human listeners to hear. Zebra finches also show a similar performance asymmetry when tested on synthetic Schroeder wave motifs that have no overall spectral and envelope differences across syllables. This shows that the asymmetry in discrimination performance between syllable structure and syllable sequence obtains fully with two simple features alone: temporal fine structure within the syllable and temporal envelope of the whole motif.

The focus of this work is on the zebra finch because of its rich and fairly recent history as a model for understanding the learning and perception of sound sequences. We tested two other avian species to determine whether zebra finches were peculiar with respect to the large performance difference in discriminating reversed syllables versus shuffled syllables. Canaries, another relatively closed-ended vocal learner that makes only modest song changes from season to season, are also relatively insensitive to changes in syllable order in zebra finch motifs. Canaries tend to produce phrases consisting of repetitions of the same syllables. As far as we know, there are no equivalent psychophysical data from canaries addressing the relative salience of syllable reversals versus syllable sequence. The comparison of finches and canaries with budgerigars, however, is intriguing. In contrast to both zebra finches and canaries, budgerigars are a very open and flexible vocal learning parrot with an extremely complex and learned warble song with a limited sequential structure that can be described as a 5th order Markov sequence (Tu and Dooling 2012, 2017).

Our results show that budgerigars are quite sensitive to changes in syllable order in zebra finch motifs which typically consist of 5–7 syllables. Whether our findings represent a songbird-parrot difference or a species difference in fixed/flexible song learners cannot be addressed with only two species and such a limited sample size. However, these results do suggest an interesting avenue for further comparative investigation on the neural basis of fine structure and envelope processing in birds. Another recent perceptual study of these zebra finches and budgerigars also showed a difference in both capability and strategy in the processing of sequences of song elements (Spierings and ten Cate 2016).

In the current study, we did not control for experience in such a way (i.e. isolation-rearing, controlled acoustic exposure, controlled social access, etc.) that would allow us to unambiguously disentangle the effects of familiarity due to acoustic exposure alone versus acoustic experience with the stimuli in the context of social experience. In a limited test on the role of the total amount of acoustic exposure, several birds in these experiments were re-tested on motif sets that they were originally tested on at the beginning of these experiments. We did this as a check on whether the performance on these stimuli, especially the syllable shuffled motifs, improved over the many months of testing in which they were repeatedly exposed to both reversed and shuffled motifs and reinforced in both cases for correct responses. In all of these experiments, the birds' performance between initial tests and later tests were similar. This shows that once birds stabilized in initial training, repeated acoustic exposure in the experimental apparatus to various natural and altered motifs over the course of these experiments did not improve performance on either reversed or shuffled stimuli.

A rigorous test of whether specific acoustic exposure in a natural context directly affects the birds' performance would require a more controlled experimental design. But our observation that acoustic experience with test stimuli in a social context appears to improve a zebra finches' ability to discriminate manipulations in syllable order is certainly consistent with the widely held notion that social experience can affect complex auditory perception. It is worth noting that there is a growing literature that describes the importance of social experience for song learning in birds (Chen et al. 2016; Baran et al. 2017; Beecher 2017), as well as, of course, for language learning in humans (Doupe and Kuhl 1999; Goldstein et al. 2003; Kuhl 2010). There is also a robust line of research that focuses on describing potential neural mechanisms that support experience-dependent changes in auditory processing (Hauber et al. 2013; Ikeda et al. 2015; Mouterde et al. 2017; for reviews, see Bolhuis and Eda-Fujiwara 2003; Bolhuis and Gahr 2006).

In songbirds, complex auditory discrimination involves a number of different brain areas likely including nuclei within (1) the song control system (SCS) which is composed of motor control areas HVC (proper name) and the robust nucleus of the arcopallium (RA) as well as the anterior forebrain pathway, including the lateral portion of the magnocellular nucleus of the anterior nidopallium (LMAN) and Area X; and (2) the avian equivalent of the auditory cortex, including the caudal medial nidopallium (NCM) and caudal medial mesopallium (CMM). Importantly, it is unrealistic to think that the physiological responsiveness of any one nucleus would accurately mirror the behavioral responsiveness of the whole organism. However, neurons in HVC and in nuclei in the anterior forebrain pathway respond robustly to playback of bird's own song (BOS) and there is almost no response following playback of the full spectro-temporal reversal of BOS (Margoliash 1983; Doupe and Konishi 1991; Janata and Margoliash 1999). The full reversal of BOS involves gross manipulations to both syllable structure and syllable order, making it hard to determine exactly which acoustic manipulation in the BOS is responsible for the difference in neural responsiveness between the BOS and the full spectro-temporal reversal of BOS. The fact that there is only a slightly less robust response to reverse ordered BOS song, compared to the BOS, is consistent with the growing notion that zebra finches are particularly sensitive to the acoustic structure of syllables, as opposed to sequences of syllables, at least as is suggested by neurophysiological studies in both the SCS and auditory regions (Coleman and Mooney 2004; Doupe 1997; Peh et al. 2015). However, Lu and Vicario (2014) clearly showed that neurons are responsive to the transition probabilities between synthetic zebra finch-like syllables.

The general finding from Experiment 3, that the discrimination of synthetic motifs where manipulations of only two acoustic features (fine structure and temporal envelope), largely reproduced behavioral discrimination data from natural motifs is particularly important. These results suggest such synthetic stimuli might be especially useful in probing brain nuclei in the auditory and song circuits more precisely in order to understand how these two prominent, independent, acoustic features of zebra finch song motifs are coded, learned, and perceived.

Perhaps the most significant outcome of these experiments are the implications of the relative salience of syllable structure and syllable order for zebra finch natural communication. Our results suggest that zebra finches may be communicating more important information through the acoustic fine structure of individual syllables than they are with syllable order (Dooling and Prior 2017; Prior et al. in press). In one sense, since the zebra finch song has a fixed syllable sequence throughout life, it would not be surprising that this is not a flexible communication channel for these birds, as for instance, the way that word order is for humans. On the other hand, the increase in sensitivity to changes in syllable order in familiar song certainly suggests that sequential information may come to have communicative significance with partners or other members of the flock. Determining the mechanisms underlying this plasticity is an important challenge. One possibility is that the same mechanisms control production and perception of sequences and an insensitivity to order may be a developmental byproduct of the dampening of sequential variability in production. If so, perhaps song perception crystallizes along with song production. Braaten et al. (2006) found that with extensive training, juvenile finches could categorize normal motifs and syllable order-reversed motifs but not at a high level. It would be interesting to compare adult zebra finches and juvenile finches, who have yet to crystalize their song, on the relative discriminability of syllable structure and syllable order in future experiments.

The tractability of zebra finches for both electrophysiological and behavioral investigations and the fact that they learn the sequences of syllables in their song have invited speculations into their ‘syntactic’ abilities and the neural coding of song sequences (Bolhuis and Eda-Fujiwara 2010; Berwick et al. 2011; Pfenning et al. 2014). To be productive, any study of sequences of sounds, human or animal, would require some understanding of the perceptual units of the acoustic communication stream. In the case of human language, the units of speech seem obvious though there has long been a debate about exactly what the acoustic-perceptual units of speech (phoneme, syllable, word, etc.) are and the extent to which bottom-up versus top-down processes are involved in processing these acoustic-perceptual units (Kazanina et al. 2006; Kazanina et al. 2017). The present results should remind us that it is not always clear what the perceptual units for acoustic communication are in birds. Traditionally, there is general agreement that notes (continuous traces on a sonogram) and syllables (recurring collection of notes) form the basic units of song and these basic units can be concatenated into larger units of song (Catchpole and Slater 2008). These “units” are historical in bird work in part because they are so obvious to the human ear. Much of the recent syllable sequence work with song birds from the laboratory, such as starlings, Bengalese finches, and zebra finches in particular, involves manipulations of sound sequences at the level of the syllable. There is no disagreement that birds can learn to categorize different syllable sequences (e.g., ten Cate and Okanoya 2012), although the number of trials involved in these categorization studies is usually quite large, raising questions of how perceptually salient syllable order really is in these different sequences. Furthermore, Beckers et al. (2017) raised questions about what exactly animals learn in artificial grammar studies. Many of these studies have been done in zebra finches, and it is sometimes difficult to determine whether ‘syntactical’ cues underlie the categorization of grammar-consistent versus grammar–violating test strings of acoustic tokens. These findings all suggest considerable differences in the processing of sequences of sounds by zebra finches and humans.

On the other hand, the potential effects of familiarity that we observed are quite interesting and in some ways are reminiscent of some of the classical cross-cultural differences in speech perception capabilities in humans where early experience with speech sound contrasts such as /Ra/-/La/are critical for later discrimination and categorization (e.g., Miyawaki et al. 1975). Perhaps exploring the mechanisms underlying this effect of experience on the sensitivity to syllable sequence in both male and female zebra finches could have broad implications for other vocal learning systems, including human language.

The focus on the unexpected poor sensitivity to sequence discrimination should not overshadow the more surprising, and perhaps more relevant, finding of an extreme sensitivity to changes in the temporal fine structure in these complex vocalizations. There is increasing evidence of the importance of temporal fine structure for speech perception and speaker identification in humans (e.g. Moore 2014), but the present results go beyond this in terms of precision. Recent work now confirms that zebra finches are able to hear the slightest within-period change in temporal fine structure that the vocal tract is capable of producing (Prior et al. in press). There are two profound conclusions from these results. One is that zebra finches have the capability to exploit an enormously rich acoustic communication channel in the acoustic fine structure of their vocalizations which heretofore has been largely ignored in studies of zebra finch acoustic communication. The other conclusion is that the ability of finches to hear this level of acoustic fine structure in their vocal signals calls into serious question whether human hearing can be used as a gauge of which aspects of complex song are perceptually relevant to birds. It is likely, as in the case of the perception of syllable structure versus syllable sequence in zebra finches in particular, that this may be leading us away from the most important channel that birds may be using in acoustic communication. At the very least, our results suggest that a full appreciation of the richness of the zebra finch acoustic communication system is going to require a much more complete understanding of the acoustic nuances and perceptual consequences of syllable structure as opposed to the relative salience of changes in syllable sequence. This is the case, even though on the surface, syllable sequence has more of an obvious parallel to other complex acoustic communication systems like human language.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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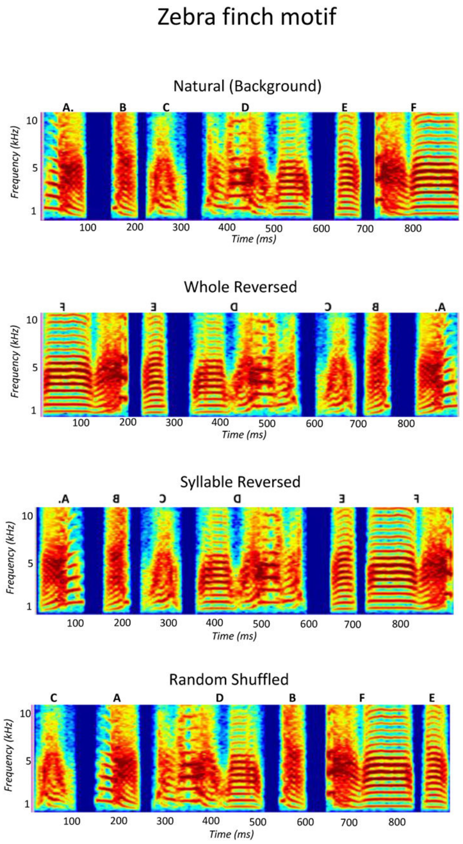


**Figure 1.**

An example of four versions of a single zebra finch motif. The forward natural motifs (illustrated in the top row) were always used as the background while targets consisted of seven modifications of this natural motif of which three are shown here including completely reversed motif (2nd row), a syllable-reversed motif (3rd row), and a randomly shuffled syllable motif (4th row). Syllable-reversed motif targets reverse the acoustic structure of each syllable while maintaining syllable order in the motif. Order-reversed motif targets reverse the sequence of syllables but preserve syllable structure.

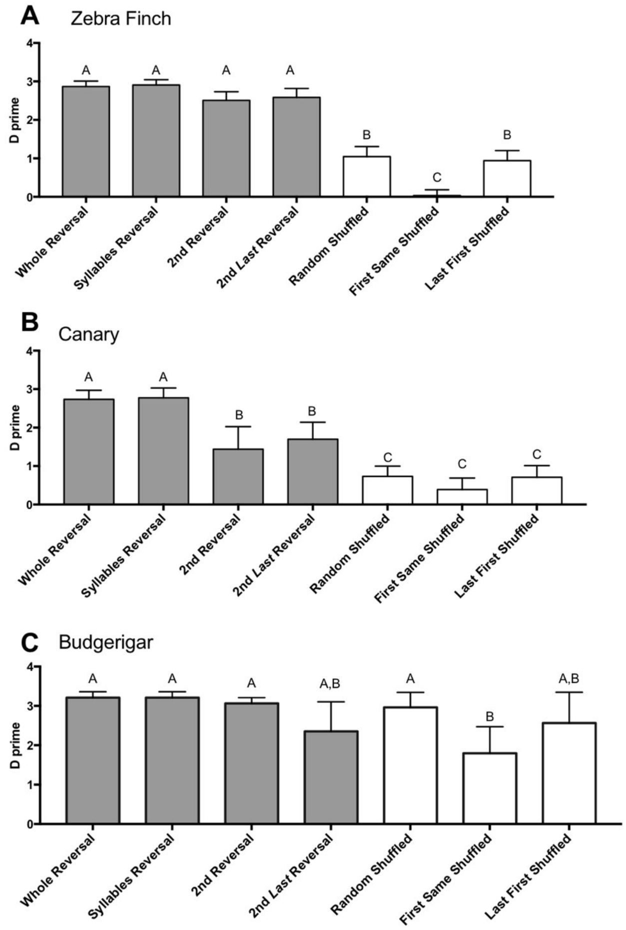


**Figure 2.**

Average d’ and standard errors for (A) 7 finches, (B) 3 canaries, and (C) 2 budgerigars on each type of motif modification. Performance was much better on all of the motif modifications involving syllable reversals (gray bars) than on those involving shuffled syllables (white bars). For the shuffled syllable motifs, performance was worst on modified motifs where the first syllable remained the same and the rest were shuffled. Results of the Tukey’s HSD follow up test are indicated with letters. (Average ± SEM)

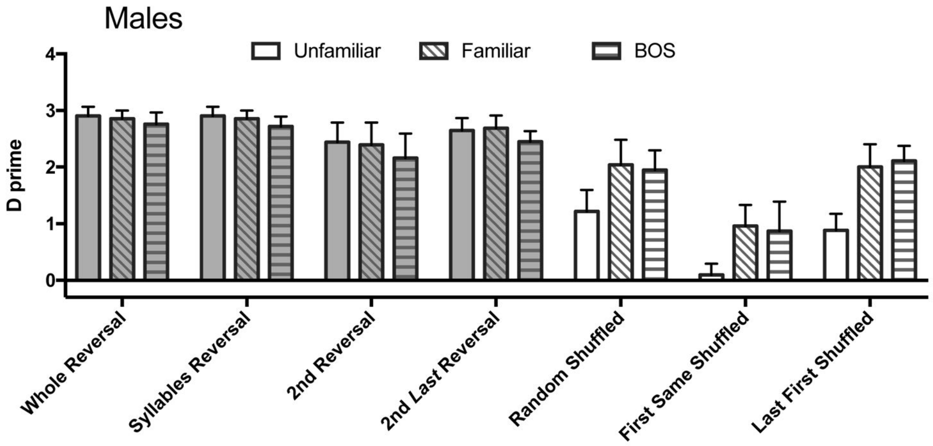
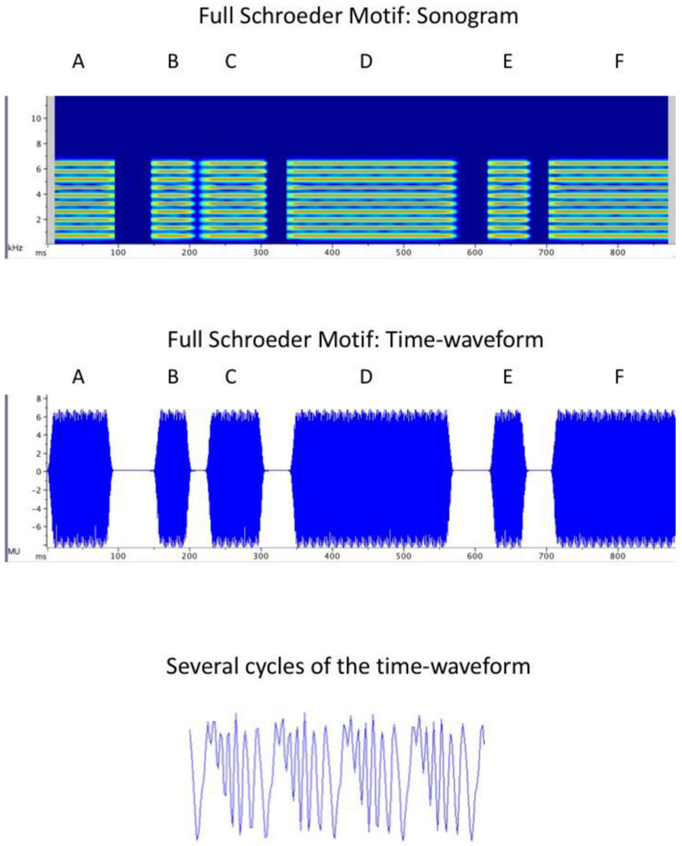


**Figure 3.**

Discrimination of reversed (gray bars) and shuffled motifs (white bars) of unfamiliar, familiar (indicated by bars with diagonal hatches), and BOS (indicated by bars with horizontal hatches motifs by four male zebra finches. The four male finches discriminated reversed motifs equally well across conditions, but were much better at discriminating shuffled motifs when the motifs were from their own song or from the songs of familiar birds. Importantly, females (not shown), also had improved performance on familiar shuffled targets. (Average ± SEM)



Full Schroeder Motif: Sonogram

Full Schroeder Motif: Time-waveform

Several cycles of the time-waveform

**Figure 4.**

Top. A spectrogram of the synthetic zebra finch motif made up of Schroeder wavefoms. Middle. The time waveform of the synthetic Schroeder motif. Bottom. A few periods of the waveform. The entire motif is made up of exactly the same periods.

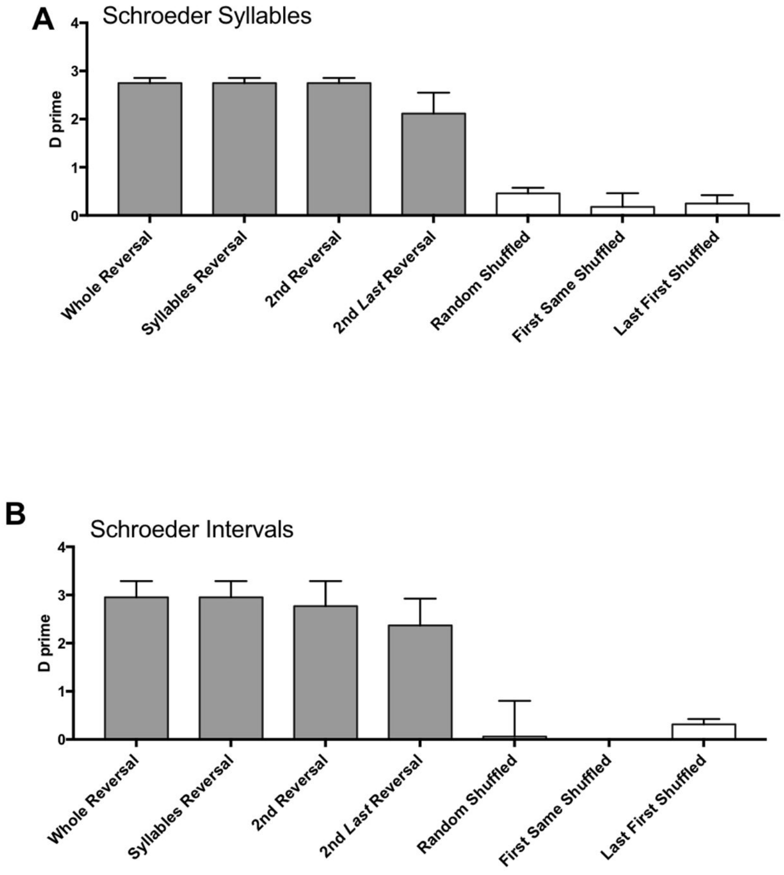


**Figure 5.**

Average d’ for three finches tested on synthetic syllable reversals (gray bars) and syllable shuffles (white bars). Birds were able to discriminate motifs that were entirely reversed, syllable reversed, or contained a single reversed syllable. Results of the Tukey’s HSD follow up test are indicated with letters. (Average ± SEM)