Sequential learning from multiple tutors and serial retuning of auditory neurons in a brain area important to birdsong learning

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ABSTRACT

Songbirds hear many vocal models during a juvenile sensitive period, transiently imitating some while retaining imitations of others in their repertoires. Despite subsequent conflicting experiences, early experience can exert lasting effects on neural structure and function, raising the possibility that transiently expressed vocalizations or their relevant models are stored in the adult songbird’s brain. One site where learned song representations could be stored is the lateral magnocellular nucleus of the anterior nidopallium (LMAN), which in the adult songbird contains neurons responsive to playback of the bird’s own song (BOS) and the tutor song (TS). To test whether LMAN neurons develop and retain responses to transiently learned songs, we exposed zebra finch hatchlings (post hatch day 0 (PHD0) to a first TS (TS1) for ~30 days, isolated them for ~30 days, then exposed them to a second TS (TS2) for 30 days starting at PHD 60. Behavioral analysis showed that PHD60 juveniles had started to copy TS1, although this copying was transient, because the adult BOS resembled TS2 and not TS1. We found that LMAN auditory responses paralleled these behavioral changes: LMAN neurons at PHD60 responded strongly and selectively to both the juvenile BOS and TS1, while LMAN neurons in adults responded to the adult BOS and TS2, but not to the transiently learned song or its model. Therefore, LMAN auditory responses can be lost or overwritten as the juvenile copies a new song, suggesting that the adult LMAN does not store information about transiently learned songs or their models.

Key Words: zebra finch, auditory selectivity, intracellular
INTRODUCTION

Conflicting experiences in the juvenile can result in multiple sensory representations and enhanced behavioral capabilities in the adult (Feldman and Knudsen 1997; Knudsen 1998; Tees and Werker 1984; Udin 1983). Unlike most animals, songbirds learn their vocal repertoires by imitating vocalizations heard during a juvenile sensitive period (Doupe and Kuhl 1999; Marler and Peters 1987). Of many models heard, some are imitated only transiently while others persist in the adult repertoire (Marler and Peters 1981). This study addresses whether songs transiently learned by juvenile zebra finches evoke auditory responses in a nucleus important to song learning.

The telencephalic nucleus LMAN is necessary for juvenile song learning (Bottjer et al. 1984; Scharff and Nottebohm 1991) and adult forms of audition-dependent vocal plasticity (Brainard and Doupe 2000) (Figure 1). LMAN neurons exhibit both auditory and vocal-related activity (Doupe and Konishi 1991; Hessler and Doupe 1999), and juvenile auditory and vocal experience influences the structure and function of LMAN neurons. Tutor song experience acts on or through LMAN, because raising birds in isolation from a tutor slows LMAN synaptic maturation (Livingston et al. 2000; Wallhausser-Franke et al. 1995) and blocking NMDA-receptors in LMAN during tutoring impairs copying (Basham et al. 1996). Auditory and vocal experience also strongly influences the response properties of LMAN neurons, which are highly selective for playback of the BOS and TS versus other conspecific songs (Doupe and Konishi 1991; Solis and Doupe 1999). Auditory experience of TS and auditory-vocal experience of BOS shapes these responses, because juveniles that sing disrupted songs due to vocal nerve section contain LMAN
neurons responsive to both TS and BOS (Solis and Doupe 1999). Notably, after these birds mature, BOS and TS playback largely fail to evoke suprathreshold responses from LMAN neurons, even though these stimuli activate areas presynaptic to LMAN (i.e., area X) (Solis and Doupe 2000). In other systems, synapses patterned by early experience are functionally suppressed when in conflict with subsequent experience (Brickley et al. 1994; Zheng and Knudsen 1999), raising the possibility that LMAN auditory responses remain subthreshold when the BOS conflicts with the stored model.

Conflicts between the current BOS and TS representations could arise naturally when juvenile birds sequentially copy from multiple tutors. Sequential exposure to and copying from multiple tutors may better represent learning in the wild, where juvenile birds associate with adult males other than their father (Immelmann 1969). However, prior studies of LMAN auditory tuning subjected birds to a single tutor, leaving unresolved LMAN’s auditory properties when birds copy multiple tutors. We sequentially exposed juvenile zebra finches to two tutors (Fig. 1) to test whether early experience of self-generated vocalizations or a model song is permanently engrained in the auditory responses of LMAN neurons. We used in vivo intracellular recordings from juvenile and adult LMAN neurons to detect sub- and suprathreshold responses to transiently experienced songs. We find that sequential copying of two different models is paralleled in LMAN by transiently expressed auditory responses to the first TS and the bird’s copy of this model, which are lost or overwritten as the bird learns a new song.
MATERIALS AND METHODS

Behavioral and electrophysiological techniques have been extensively described in previously published studies (Livingston et al. 2000; Rosen and Mooney 2000) and so are described more briefly here. All experiments were performed in accordance with a protocol approved by the Duke University Instructional Animal Care and Use Committee.

Sequential tutoring protocol

Thirty four male zebra finches (*Taeniopygia guttata*) were reared using a protocol that carefully controls auditory and social exposure to adult tutors, and that has been shown previously to successfully extend sensory acquisition beyond PHD 60 (Livingston et al. 2000). Twenty-three of these experimental birds were hatched and reared until PHD 22-36 (28 ± 0.82 PHD, n = 23) with their father (Zebra finch-Fathered birds: ZF-birds), mother and siblings in a sound isolation chamber (Industrial Acoustic, Bronx, New York). Eleven of the 34 experimental birds were reared initially by Bengalese finch (*Lonchura striata*) foster “parents” until PHD 25-35 (Bengalese finch-Fathered birds: BF-birds; 31 ± 1.3 days; n = 11 BF birds). The Bengalese finch song differs from zebra finch song in numerous ways, including shorter duration of individual syllables, a tendency to produce notes with broadly spaced harmonic elements, a higher incidence of single syllable repeats, and a variable syllable sequence. Therefore, by using a Bengalese finch as a foster father we could enlarge the acoustical differences between the first and second tutor songs, making it easier to assess which tutor supplied material copied
in the adult song of the sequentially tutored bird. Beginning PHD 22 to 36, individual birds were placed in a small cage, with two to six of these cages housed in a sound isolation chamber. During this period, BF birds and ZF birds were never housed in the same isolation chamber. Opaque partitions were placed between individual cages. Juveniles housed in this manner could hear but not see each other, and were totally isolated from adult birds of either sex. Under these conditions, juvenile zebra finches do not copy from each other (Livingston et al. 2000; Morrison and Nottebohm 1993).

Although birds were isolated from the first tutor across a fairly wide age range (i.e., PHD 22-36), no correlation was detected between the age of isolation from the first tutor and the acoustic similarity of the BOS at PHD60 and TS1 using either Sound Analysis or subjective scoring methods (data not shown; see below for song analysis methods). Birds remained in visual isolation until PHD 60-65, when they were either used for experiments or subjected to a second tutor. Twelve birds were either used for electrophysiology at PHD 60 (7 ZF and 1 BF), or otherwise were not subjected to a second tutor, and used strictly for behavioral analysis (4 BF birds). The remaining twenty-two birds were paired with a second tutor (an adult male zebra finch distinct from the biological or foster father) starting at PHD 60 (mean = 61.3 ± 0.42) for 20-34 days (mean 24.1 ± 0.94 days), and then isolated again until used for electrophysiological recordings after PHD 115 (mean = 139 ± 4.8) (Fig. 1A). Adult birds that had been sequentially tutored by two zebra finches are termed “ZZ” birds (n =16), whereas adult birds initially tutored by a Bengalese finch and then by a zebra finch are termed “BZ” (n = 6) birds. See table 1 for the behavioral analysis of these 34 birds.
Behavioral Analysis

Songs were recorded at approximately one-week intervals beginning just prior to introduction of the second tutor (~PHD 60) for one month, and then approximately biweekly continuing up to the day prior to electrophysiological recording. This recording schedule was followed because we observed that the juvenile bird’s songs changed dramatically in the first two weeks of the second tutoring period, and then changed more gradually towards the end of this tutoring period and throughout the second isolation period. To obtain song, the bird was placed in a small recording chamber with an adult female except during the second tutoring period, when the bird was recorded in the presence of its tutor. We observed that the time juveniles started to sing after introduction to the recording chamber could be quite variable, from several hours to more than a day. Therefore, we recorded the bird’s song during the second tutoring period in the presence of the second tutor, to minimize interindividual variation in the amount of total tutor exposure. All songs were recorded using a Shure SM-57 microphone placed ~25 cm in front of the bird’s cage, the signal from which was low pass filtered at 10 kHz, sampled at 22.05 KHz using a data acquisition card (National Instruments AT MIO 16E Series) and written directly to disk using software written in the LabView environment by Ramani Balu. The songs from the tutor birds, as well as other unrelated conspecific songs (CON), were recorded in the presence of an adult female. Prior to electrophysiological experiments in adult birds, songs were recorded at least twice over an interval of one or more weeks to ensure that birds were singing stable songs. Final songs were recorded within the twenty-four hours preceding the experiment.
We used ‘Sound Analysis 2’ (SA) (Tchernichovski et al. 2000) to measure the overall similarity of the BOS at the final adult point (BOS\textsubscript{Adult}) (i.e., at the time of electrophysiological recordings we made in adult birds, range 117 - 205 PHD; mean = 139 ± 4.8; n = 22 birds subjected to electrophysiological recording) to juvenile versions of the BOS (i.e., BOS at PHD60 (BOS\textsubscript{PHD60}) or to either of the tutor songs (TS1 and TS2), as well as CON songs. When comparing the song of a subject zebra finch to another zebra finch song, we recorded more than 10 renditions of each song and picked the most frequently repeated single motif. We compared single motifs from each of the two songs (excluding introductory notes). The various parts of a given motif were identified automatically by periods of silence greater than 70 msec, and then were compared to the parts of the other motif (SA settings: “chunk-to-chunk” mode, threshold 81%, interval 70 msec). In Sound Analysis 2, song similarity is automatically calculated by estimating and comparing 4 acoustic parameters in the two songs: pitch, entropy, frequency modulation (FM) and continuity. Here we report the ‘similarity’ scores based on these four parameters. For comparisons of juvenile zebra finch songs in which the first tutor was a Bengalese finch, we estimated the average similarity between a BOS\textsubscript{PHD60} and each of three renditions of the Bengalese tutor song, because the motif was not clearly defined in Bengalese finch song. In addition, because Bengalese finch songs comprise more tonal syllables of short duration compared to the zebra finch, we used different settings for the interval (39 msec), although the same threshold setting (81%).

Sound Analysis is insufficient by itself to assess low levels of copying, as two songs could
achieve a low similarity score either because they were unrelated (i.e., not derived from the same tutor) or because one was modeled only in part after the other. Therefore, to better assess copying, we used subjective scoring by four observers experienced in birdsong analysis who were asked to compare each of the two tutor songs with BOSPHD60 and BOSAdult. The observers were blind to the order of the two tutors (i.e., which was TS1 or TS2). Observers were instructed to inspect sonograms and listen to each of the four songs as needed, then score those syllables of the BOSAdult or BOSPHD60, if any, that were similar to those found in either of the tutor songs. The criterion for deeming that a syllable had been ‘copied’ was that at least three of the four observers scored a syllable of BOSAdult or BOSPHD60 as copied from either of the two tutors. Percent similarity of song was calculated from the number of syllables copied from a given tutor divided by the total number of syllables in the BOSAdult.

Song stimuli for electrophysiological recordings

All songs were low-pass filtered at 10 kHz, digitized at 22.05 kHz and edited to include 2 or 3 motifs for each song. Song stimuli included the BOSAdult, BOSPHD60, TS1 and TS2. Most cells were presented these four songs in both the forward and reverse (Song-REV) direction, and also presented a CON song. We used a total of 5 different CON songs for all of the electrophysiological experiments performed here, although we used the same CON song consistently throughout a single experiment. Each stimulus was played through a speaker facing the bird at a distance of ~25cm and was 1.5-2.5 sec in duration, with an average sound pressure level of 70dB (RMS, A-weighting). The various song
stimuli were presented in a fixed sequence during a recording from a given cell.

*In vivo electrophysiology*

Of the thirty four birds used in this project, twenty-two (16 ZZ and 6 BZ; 1 ZZ bird failed to yield any responsive LMAN neurons) were used for electrophysiological recordings after PHD 115, and eight (7 ZF and 1 BF) were used for electrophysiological recordings at the end of the first isolation period (PHD 62-69; i.e., they were not exposed to a second tutor). On the day of the electrophysiological experiment, the bird was injected intramuscularly with 20% urethane in dH₂O (90-100 µl total; Sigma, St. Louis, MO) in 30-40 µl doses at 30 minute intervals, further anesthetized with inhalation anesthetics as needed, and then equipped with a stainless steel post mounted on its head with dental acrylic and cyanoacrylate.

Sharp electrodes (borosilicate glass, BF100-50-10; Sutter Instrument, Navato CA) were pulled to 60-200 MΩ when filled with 2M K-acetate, 5% neurobiotin, and placed into the nucleus using stereotaxic coordinates and a hydraulic microdrive (Soma Scientific). An AxoClamp 2B intracellular amplifier (Axon Instruments) recorded intracellular potentials in bridge mode. The recorded potentials were low-pass filtered at 3 KHz, digitized at either 10 or 11.05 KHz (National Instruments PCI MIO 16E series Data Acquisition Board) and stored on a PC. In the first several experiments, we used intracellular staining with neurobiotin to confirm post hoc that we were indeed recording from LMAN neurons. In subsequent experiments, LMAN neurons were identified on-line by their characteristic
spike shape and firing patterns (Livingston and Mooney 1997; Rosen and Mooney 2000). If the impaled LMAN neuron had a resting membrane potential negative of -60mV, and also displayed robust spontaneous synaptic activity, we then probed its auditory response properties using the panel of song stimuli mentioned above. In some cases, tonic negative or positive currents were injected through the electrode to shift the membrane potentials of the neurons, to accentuate evoked synaptic or action potential activity, respectively. In these cases, comparisons of responses to different stimuli were made at the same membrane potential. Ten to ninety iterations of each auditory stimulus, including 2 seconds of silence pre- and post-stimulus, were delivered at 5 second intervals to the adults and 10-12s intervals to the young birds via a speaker ~ 25 cm in front of the bird’s head. Typical experiments lasted ~ 12 hours; although robust responses usually persisted over this duration, experiments were discontinued if responsiveness to the BOS diminished or disappeared.

Analysis of electrophysiological data

For analyzing electrophysiological data, we used methods that have been described in previously published studies (Rosen and Mooney 2000). The suprathreshold responsiveness of neurons was calculated by response strength (RS) = $S_{FR} - B_{FR}$, where $S_{FR}$ and $B_{FR}$ are the firing rates during each whole song stimulus presentation and during the baseline period before each song stimulus presentation, respectively. To determine whether a suprathreshold response was significant for each cell, we used a paired t-test to compare mean firing rates during each song stimulus presentation and
during a pre-stimulus baseline period of equal duration, and $p < 0.05$ as the criterion for responsiveness.

For measuring the subthreshold response, we calculated a $z$-score for positive area ($Z_{area}$): The area measurements were given by the difference between the average area below median filtered traces during song stimulus presentation ($S_{area}$) and during the period immediately prior to each song stimulus presentation ($B_{area}$), each calculated relative to the baseline membrane potential value estimated by the mode of the pre-stimulus resting potential.

$$Z_{area} = (S_{area} - B_{area}) / \sqrt{\text{Var}(S_{area}) + \text{Var}(B_{area}) - 2\text{Covar}(S_{area}, B_{area})}$$

To maximize the possibility of detecting phasic responses to song playback, we also calculated the $Z_{area}$ of the response peak: A window (200-800 ms wide) was centered about the highest peak during song presentation, and a window of equivalent size was centered about the largest pre-stimulus peak. The largest peaks in the median-filtered trace averages were determined by visual inspection.

To determine whether a neuron was responsive to a given song at the subthreshold level, we used a paired $t$-test to compare average area during the peak of each song stimulus presentation and during the peak of a pre-stimulus baseline period of equal duration, and $p < 0.05$ as the criterion for significance.

To compare the relative response bias to BOS versus one of the other song stimuli (reversed BOS (REV), BOSPHD60, TS1, TS2 and CON), we used a $d'$ statistic, which estimates the discriminability between two stimuli for an ideal observer (Green and Swets 1966):

$$d'_{FiringRate} = 2(R_{SFRBOS} - R_{SFRsong}) / \sqrt{(\sigma^2_{BOS} + \sigma^2_{song})}$$

$R_S$ is the mean value of $S_{FR} - B_{FR}$, and $\sigma^2$ is the response variance for the given stimulus. A $d' > 0.5$
reflects a net bias to the BOS, whereas a \( d' = 0 \) reflects no net bias. This measure is superior to a ratio measure of response strengths because it takes into account the variance of the responses and can report negative values (Solis and Doupe 1997).

Statistical tests: In those cases where multiple comparisons were made, an ANOVA was used followed by a post hoc mean comparison using Tukey’s test, with significance determined at \( p < 0.05 \). Other specific tests used to determine significance are given in the results and/or figure legends. All values reported are mean ± SEM, unless otherwise noted.
RESULTS

The first section of the results addresses the behavioral analysis of song learning in juvenile and adult birds raised in the sequential tutoring protocol. The second section of the results describes the electrophysiological response properties of LMAN neurons to song playback in these juvenile and adult birds.

BEHAVIOR

*Songs of PHD 60 juveniles raised in a sequential tutoring protocol*

To distinguish whether copying from TS1 had occurred at the end of the first isolation period (PHD 60), we analyzed song of 15 birds where another zebra finch was the tutor during the first posthatch month ("ZF" birds; note that song of one ZF bird was not recorded at PHD60, so this bird was only used for the adult song comparisons). Although song structure is only roughly formed at this stage, complicating subjective analysis, blind observers still identified song syllables similar to syllables in the songs of the first tutor in three of the fifteen juveniles (Fig. 2; Table 1; mean % similarity = 4.1 ± 2.2 %, n = 15 birds; copied syllable range 0-1, mean 0.2 ± 0.11; total syllable range 2-9 syllables, mean 4.5 ± 0.42 syllables). In contrast, blind observers never scored songs of PHD 60 birds as similar to the second tutor, a bird to which the PHD 60 juveniles had not yet been exposed (Fig. 2C; note that for subjective scoring at this stage the “CON” songs were TS2). A further indication of copying from the first tutor was provided by percent similarity scores calculated using Sound Analysis, an automated routine that estimates similarity between two motifs using four acoustic parameters: entropy, pitch,
frequency modulation and continuity (Tchernichovski et al. 2000). We found the similarity between BOSPHD60 and TS1 was significantly higher than between BOSPHD60 and TS2 (i.e., a zebra finch song to which they had not yet been exposed) (BOSPHD60 and TS1 50 ± 6.9 %, BOSPHD60 and TS2, 35 ± 6.3 %, p <0.02, paired t-test; n = 15). In addition, the mean similarity between the BOSPHD60 and TS1 was slightly but not significantly greater than the mean similarity between the BOSPHD60 and any of four different CON songs (Fig. 2C; p < .05, ANOVA). Thus, analysis of song prior to introduction of the second tutor provides evidence of partial copying from the first tutor, at least in some birds.

**Juvenile copying from Bengalese finch TS1**

A potential limitation of using zebra finches as the first tutor in the sequential tutoring protocol is that a relatively high degree of similarity between the pupil and tutor song could arise by chance (in our experience, the percent similarity for songs of unrelated adult birds from our colony was > 30 %, when using Sound Analysis; Fig. 2C). This chance similarity may further confound an assessment of copying at PHD 60, because juvenile zebra finch songs are still relatively unrefined at this time. Although our prior analysis of zebra finch song indicated that several PHD 60 birds had copied from the first of their two zebra finch tutors, we wanted to search for clearer evidence of this early copying under conditions where the level of chance similarity was reduced. In contrast to zebra finch songs, Bengalese finch songs consist of relatively short duration syllables with often broadly spaced harmonic structure; these syllables are repeated a varying number of times in a given Bengalese finch motif, in
distinction to zebra finch motif structure (Fig. 3A). These acoustic differences lower the likelihood that similarities between a juvenile zebra finch and its Bengalese finch tutor would arise by chance (the mean song similarity between zebra finch song and Bengalese finch song in our colony is $16.4 \pm 3.3\%$, as estimated by Sound Analysis). Therefore, we assessed the songs of PHD 60 zebra finches that were raised with Bengalese finches for the first posthatch month.

We found that the songs of juvenile zebra finches tutored by Bengalese finches showed clear evidence of copying from their Bengalese finch tutors at PHD 60, as indicated by both blind subjective analysis and Sound Analysis (Fig. 3A, B). Subjective analysis indicated that eight out of eleven zebra finches tutored initially by a Bengalese finch had copied one or more syllables from this heterospecific tutor (Fig. 3B; Table 1; mean % similarity = $15.4 \pm 5.1\%$; mean number of syllables copied at PHD60 = $1.1 \pm 0.28$; total syllables $\text{BOS}_{\text{PHD}60} = 7.4 \pm 1.1$; $n = 11$ birds). Other juvenile song features suggestive of copying from the Bengalese tutor were the significantly higher total syllable number and shorter syllable durations compared to songs of those birds raised initially with a zebra finch tutor (Table 1; $7.4 \pm 1.1$ versus $4.5 \pm 0.31$ total syllables, $p = 0.02$; $51.8 \pm 2.8$ versus $78.9 \pm 4.8$ msec syllable duration, $p < 0.0001$; Welch's t-test, $n = 11$ BF and 22 ZF birds). In addition, mean similarity scores between the $\text{BOS}_{\text{PHD}60}$ and Bengalese finch tutor songs calculated using Sound Analysis were higher than those obtained between the same juvenile songs and songs of non-tutor zebra finches (Fig. 3C; $\text{BOS}_{\text{PHD}60}$ - TS1, $44.5 \pm 8.3\%$ versus $\text{BOS}_{\text{PHD}60}$ - CON, $24.9 \pm 2.8\%$; $p < .05$ as determined by an ANOVA). These results suggest that birds reared in the first stage of our sequential tutoring protocol copy syllables
from the first tutor by PHD 60.

**Adult songs of sequentially tutored birds**

Adult birds that had been sequentially tutored as juveniles by two birds showed strong evidence of copying from the most recent tutor (TS2), and little or no sign of copying from TS1 (Fig. 3A, 4A). We exposed a total of 22 zebra finches to a second (zebra finch) tutor starting at 60 days (mean 61.3 ± 0.42 PHD; 16 birds were initially tutored by another zebra finch (ZZ birds), and another 6 birds were initially tutored by a Bengalese finch (BZ birds)). Syllable copying from the second tutor, as assessed by subjective scoring, was observed in 14/16 adult ZZ and 4/6 adult BZ birds (Fig. 4B). For all twenty-two birds, we found that on average 2.9 ± 0.4 out of 6.2 ± 0.4 BOS syllables were copied from TS2 (Table 1), yielding a mean subjective similarity score of 48.7 ± 7.0 % (Fig. 4). Automated scoring of similarity between BOS\textsubscript{Adult} and TS2 using Sound Analysis, showed that the BOS\textsubscript{Adult} was significantly more similar to TS2 than to the songs of non-tutor (CON) zebra finches (61.5 ± 5.6% versus 36.3 ± 2.2%, each BOS\textsubscript{Adult} was compared to the songs of four non-tutor zebra finches from our colony). The similarity scores between BOS\textsubscript{Adult} to TS2 obtained using Sound Analysis (> 60% similarity) were comparable to the similarity scores others have obtained when comparing an adult bird’s song to that of its tutor (Tchernichovski et al. 2001).

The quality and amount of copying from TS2 approached values obtained in other studies where isolation was used to extend the period of song memorization (Aamodt et al. 1995; Eales 1985;
Although the number of syllables copied from TS2 varied across birds (as estimated by subjective scoring), five (4 ZZ and 1 ZZ) of the twenty birds copied almost all (> 80%) of their syllables from their second tutor (see examples in Fig. 3A and 4A). In those birds that copied two or more of their syllables from TS2, the syllable sequences were never altered from those of the tutor (Fig. 3A, 4A). The amounts of copying as well as the tendency to maintain syllable sequences in their original order are features typical of the song learning process in zebra finches (Immelmann 1969).

We found little evidence that adult birds retained syllables from their first tutor following exposure to a second tutor. Over 80% (18/22) of the sequentially tutored adult birds showed no evidence of syllables copied from TS1 (Fig. 4B, right; 4/22 birds retained syllables from TS1: one ZZ bird copied one syllable, one BZ bird copied two syllables, and two BZ birds copied one syllable; based on subjective scoring). Furthermore, estimates of song similarity calculated using Sound Analysis indicated that the mean similarity between BOS_{Adult} and TS1 was not significantly different from the similarity between BOS_{Adult} and CON (48.7 ± 7.0 % versus 36.3 ± 2.2 %; NS, ANOVA). However, the incidence of high similarity scores (i.e., > 50% similarity) in the BOS_{Adult} and TS1 comparisons (8/22 birds) was slightly higher than the range of high similarity scores in other BOS_{Adult} and CON comparisons (BOS_{Adult} > 50% similarity to 4 CON songs ranged from 3-7 out of the 22 birds, using Sound Analysis). Overall, the BOS_{Adult} tended to resemble the TS2 and not the TS1, as reflected in the mean similarity scores obtained using both subjective scoring and Sound Analysis (Subjective: BOS_{Adult}}
and TS2, 48.7 ± 7.0%; BOS\textsubscript{Adult} and TS1, 3.2 ± 1.6%; Sound Analysis: BOS\textsubscript{Adult} and TS2, 61.5 ± 5.6%; BOS\textsubscript{Adult} and TS1, 42.0 ± 5.2%).

The similarity to TS2 and not TS1 also characterized the songs of the subset of adult birds that previously had shown clear evidence of copying from their first tutor at PHD60, based on subjective analysis (n = 7; 3 ZZ and 4 BZ birds; 6/7 adult birds showed copying from TS2; average similarity for all 7 birds’ songs to TS2 using Sound Analysis was 59.8 ± 11.9 %, note that two of these birds’ adult songs were judged by subjective scoring to be slightly similar to TS1 (25% and 11 %)). These results indicate that the protocol used in these experiments produces adults with songs copied from the most recent tutor (TS2), with little or no copying from the first tutor to whom they were exposed (TS1). This tendency to copy from the most recent tutor is a trait also manifested in zebra finches tutored serially in a contiguous manner (i.e., without an intervening isolation period-see (Eales 1985)).

To further quantify how the BOS changed over development in relation to each of the birds’ two tutors, we estimated the change in similarity between the BOS and a given tutor song from PHD 60 to after PHD 115 (Fig. 5A). These developmental changes in individual birds’ songs were estimated by subtracting the similarity score between the BOS\textsubscript{PHD60} and a given tutor song (i.e., TS1 or TS2) from the similarity score between the BOS\textsubscript{Adult} and the same tutor song. Automated similarity score differences showed that the songs of 16/21 birds became more similar to TS2 after exposure to the second tutor (Fig. 5A, left). Similarly, subjective similarity score differences revealed that songs of 17/21 sequentially tutored birds became more like TS2 over this period (Fig. 5A, right; note that song from one bird was
not recorded prior to the second tutoring period). In contrast, subjective analysis indicated that similarity to TS1 either stayed the same or decreased in 18/21 birds over this same period (Fig. 5A, right). Comparisons of Sound Analysis similarity score differences also revealed that the majority (13/21) of birds’ songs decreased in similarity to TS1 over development (Fig. 5A, left). Ultimately, the mean similarity score of the BOS to TS2 estimated using Sound Analysis increased markedly between PHD 60 and 115 (33.5 ± 4.8 % similarity between BOSPHD60 and TS2 versus 61.5 ± 5.6 % between BOSAdult and TS2, p = 0.003, paired t-tests), while over the same period the BOS decreased slightly but not significantly in similarity to TS1 (48.6 ± 6.4 % between BOSPHD60 and TS1 versus 42.0 ± 5.2 % between BOSAdult and TS1; NS, p = 0.3, paired t-tests). Therefore, during development, the BOS increased in similarity to TS2, and most often decreased in similarity to TS1. This result suggests that sequentially tutored birds lost or overwrote their old songs as they learned new songs.

**Timing of BOS stabilization following introduction of the second tutor**

We also assessed the timing of changes to the bird’s own song following exposure to the second tutor. Therefore, we made a post hoc estimate of the similarity between the BOS at various times after introduction of the second tutor with the BOSAdult, recorded after PHD 115 (Fig. 5B). This age is substantially beyond that when song stabilizes in normal zebra finches (~ PHD 90) (Zann 1996), and we confirmed that song was stable after PHD 115 in these sequentially tutored birds by comparing the song at the time of sacrifice with songs produced over the prior one to three weeks. We noted that all birds’
songs changed rapidly in the first two to four weeks following the introduction of the second tutor, and were stable by the end of the second month after beginning exposure to the second tutor (the current BOS exceeded 90 % similarity to the BOS\textsubscript{Adult} 53.3 ± 5.4 days after the beginning of exposure to the second tutor). Mean similarity scores between the current BOS and the BOS\textsubscript{Adult} increased from 36.4 ± 4.9 % at the day prior to the beginning of the second tutor period (n = 21 birds; one bird was not recorded prior to the second tutoring period and two birds were not recorded during the second tutoring period), to 91 ± 3.9 % fifty three days after the beginning of second tutor period (average of 11 birds whose songs were recorded between 49 days and 56 days from the beginning of second tutor). Therefore, vocal changes including copying from TS2 occur rapidly after exposure to TS2.
ELECTROPHYSIOLOGICAL PROPERTIES OF LMAN NEURONS

Juvenile LMAN neuronal responsiveness

Prior studies suggest that LMAN neurons in juvenile zebra finches raised with normal access to a tutor are already partially selective for the bird’s own song and the tutor song by PHD60 (Solis and Doupe 1997). We were curious to know whether LMAN neurons in PHD60 birds subjected to more restricted tutor access (i.e., for only the first 35 PHD), as in the first phase of the sequential tutoring protocol used here, exhibited selectivity for their own song as well as the TS1. In the event that responses to each song might only manifest themselves at a subthreshold synaptic level, and not in the action potential discharge of LMAN neurons, we used in vivo intracellular recordings and signal averaging methods to detect both sub- and suprathreshold activity (Fig. 1B; see Methods).

We recorded LMAN neuronal activity from a set of juvenile birds at the end of their first isolation period (mean = PHD 63 ± 0.98, n = 8 birds, 7 ZF and 1 BF). At PHD 60, we found that BOSPHD60 and the tutor’s song (i.e., TS1) could elicit significant suprathreshold responses in LMAN neurons (Fig. 6A; see Methods). All 37 LMAN neurons we sampled at this time point responded to the BOSPHD60, and almost half presented with TS1 responded to this stimulus (12/25 neurons; 12 neurons in 3 birds were not tested with TS1) (Fig. 6A and Table 2). Four of five birds tested contained TS1-responsive cells; three of these four birds copied some material from TS1, while the one bird that did not contain any TS1-responsive cells also did not copy from TS1, as assessed by subjective scoring. The mean response strength was greater for the current BOS (i.e., BOSPHD60) than for all other stimuli.
tested (Fig. 6B; see figure for statistical comparisons). We also assessed the neuronal response at
subthreshold level, by calculating positive area z-scores over the duration of the entire song stimulus
($Z_{\text{area}}$; see Methods). In general, the subthreshold response properties were the same with
suprathreshold level (Fig. 6A). We encountered totally 35 neurons which showed significant
subthreshold responses. All 35 neurons responded to BOS and almost half presented with TS1
responded to this stimulus (12/25 cells; 12 neurons in 3 birds were not tested with TS1). The mean
subthreshold response z-score was greatest for the BOS and the rank order of stimulus-evoked
subthreshold response strengths was identical to the pattern seen with suprathreshold response strength
measurements (Fig. 6C). These results indicate that the current BOS and TS1 are effective at driving
sub- and suprathreshold responses in LMAN neurons of PHD60 zebra finches tutored with this protocol,
and that CON, BOS-REV and TS1-REV are largely ineffective stimuli.

One possibility is that the response strength of LMAN neurons at PHD60 to TS1 was correlated
with the acoustical similarity of this tutor song to the current BOS. However, no significant correlation
was detected when we plotted the response strength of these juvenile LMAN neurons to TS1 as a
function of the similarity between the current BOS and this model, regardless of whether we estimated
similarity using Sound Analysis (data not shown) or subjective methods (Fig. 6D). One potential
limitation is that the small number of birds may have led to an underestimation of any potential
correlation between song similarity and neuronal response.

The juvenile responsiveness to TS1 could reflect either broadly tuned properties of relatively
non-selective neurons or instead narrowly tuned properties of selective neurons. As noted previously, LMAN neurons at PHD 60 as a population exhibited strong responsiveness to the BOSP**HD**60 and TS1, but failed to respond to CON, BOSP**HD**60-REV, and TS1-REV, hinting that these neurons were already moderately selective in their response properties (Fig. 6B). We used the d’ metric to determine whether LMAN neurons at PHD60 were selective for forward playback of the current BOS versus other songs (see Methods; when using the BOS as the referent, d’ values > 0.5 reflect a biased response to the BOS over the comparison song). These d’ values indicated that LMAN neurons at PHD60 were strongly selective for the BOSP**HD**60 versus CON and BOSP**HD**60-REV (Fig. 6E, F: d’ BOSP**HD**60 - CON = 1.14 ± 0.16, n = 23, d’ BOSP**HD**60 - REV = 1.03 ± 0.14, n = 37). We also noted that LMAN neurons in these juvenile birds were selective for BOSP**HD**60 versus TS1 (d’ BOSP**HD**60 – TS1 = 0.83 ± 0.13, n = 25). Although the mean d’ value for this comparison was lower than BOSP**HD**60 versus CON, this difference was not significant (Fig. 6E). Furthermore, the cumulative distribution of d’ values comparing BOSP**HD**60 to CON, TS1 and REV were not significantly different from one another (K-S test), suggesting that LMAN neurons in juvenile birds were equivalently selective for the current BOS versus these other stimuli (Figure 6F). However, TS1-responsive neurons were selective for forward over reverse TS1 playback (Fig. 6E; d’ TS1 – TS1 REV = 0.61 ± 0.15, n = 25). Therefore, LMAN neurons in these birds were already highly selective for the current BOS over other song stimuli and showed selectivity for forward over reverse playback of the first tutor song. These results indicate that LMAN neurons in PHD60 zebra finches reared with limited exposure to a tutor nonetheless exhibit narrowly
tuned properties their own song and the tutor song.

**LMAN neuronal responses to song playback in adult sequentially tutored birds**

In other systems, patterns of early sensory experience that conflict with later experience can be maintained in a latent manner at the synaptic level (Feldman and Knudsen 1997; Udin 1983). We tested whether LMAN neurons in sequentially tutored adult birds \((n = 22)\) exhibited any responsiveness to the songs of the first tutor or to the transient copies that the juvenile had made of its first tutor’s song. In general, we found that LMAN neurons in sequentially tutored adult birds exhibited sub- and suprathreshold responses to the current BOS\textsubscript{Adult} and/or the TS2, but responded less often or not at all to either TS1 or the BOS\textsubscript{PHD60} (Fig. 7; Table 2).

In 21 of 22 birds, sub- and/or suprathreshold responses were evoked in LMAN by playback of the BOS\textsubscript{Adult} (15 ZZ and 6 BZ birds; one ZZ bird failed to yield any responsive LMAN neurons). In these 21 birds, 84 out of a total of 122 LMAN neurons showed significant suprathreshold responses to at least one of four song stimuli (BOS\textsubscript{Adult}, TS1, TS2 or BOS\textsubscript{PHD60}; see Table 2). In the remaining 38 neurons we failed to detect any song-evoked responses, and these were not included in further analyses. The vast majority of cells (73/84) responded to the BOS\textsubscript{Adult}, while approximately half (40/84 cells) responded to playback of the TS2, 16% (19/84) responded to the BOS\textsubscript{PHD60}, and 8% (9/84) responded to TS1. The responses to the BOS\textsubscript{PHD60} and TS1 were limited to five and three birds respectively. None of the three adult birds containing TS1-responsive cells had copied from TS1 at PHD60, as assessed by subjective scoring. The mean suprathreshold response strength across all sampled LMAN neurons was
significantly greater for the BOS\textsubscript{Adult} than for any of the other stimuli we used (Fig. 8A; see figure for statistical comparisons). In addition, the mean response strength to TS2 was significantly stronger than to any of the other stimuli besides BOS\textsubscript{Adult} (see Fig. 8A for statistical comparisons).

We also examined whether a strong bias to the BOS\textsubscript{Adult} and TS2 and a lack of responsiveness to TS1 characterized LMAN neurons in the subset of birds (3 ZZ and 4 BZ birds) subjectively scored as previously having copied from TS1 (i.e., at PHD60). In these seven birds, the mean suprathreshold response strength of LMAN neurons (n = 29 cells) showed the same bias as the general population of birds, with strong responses to BOS\textsubscript{Adult} and TS2 and a total absence of responses to TS1 (Fig. 8B; 0/29 LMAN neurons responded to TS1). This lack of TS1-responsiveness contrasted with the TS1-responsive LMAN neurons we encountered in PHD60 birds. This developmental difference was not due to different levels of copying in the two groups, because these seven adult birds copied 24.8 ± 6.0 % syllables from TS1 at PHD60, similar to the 22.8 ± 11.8% syllables copied from TS1 in the 5 juvenile birds from which we obtained TS1-responsive cells. Therefore, the first tutor song was an ineffective stimulus for driving suprathreshold responses in LMAN neurons in sequentially tutored adult birds that copied previously from this source.

We investigated whether “latent” synaptic responses to the TS1 or juvenile versions of the BOS might be maintained at a subthreshold level. The results of this subthreshold analysis closely paralleled the results of the suprathreshold analysis, in that both the BOS\textsubscript{Adult} and TS2 evoked a strong response, while the TS1 and BOS\textsubscript{PHD60} did not (Fig. 9A; see Figure legend for statistical comparisons). As with
the suprathreshold analysis, we did not detect any subthreshold responses to TS1 in adult birds that had copied one or more syllables from TS1 at PHD 60 (Fig. 9B).

One potential confound of this analysis is that sub- and suprathreshold responses to TS1 could be more phasic than those evoked by the BOS_{Adult} or TS2, and thus might go undetected using an analysis window spanning the entire song. Therefore, to maximize our ability to detect latent synaptic responses to TS1, we also computed a subthreshold response measured during a short time window (~200 - 800 msec) bracketing the peak response evoked by the tutor’s song. Using such a peak subthreshold analysis, we found only two additional TS1-responsive neurons, both of which came from one of the three birds in which we previously found TS1 responsive cells using suprathreshold criteria (Fig. 9C; i.e., a bird that had not copied from TS1 at PHD60, as assessed by subjective scoring). In contrast, the same analysis detected an additional 22 cells that were responsive to TS2 (Fig. 9C). Therefore, LMAN neurons in adult sequentially tutored birds rarely respond to the transiently copied tutor song, whereas a substantial portion responds to the most recent tutor song.

_Correlation between song similarity and response strength_

We hypothesized that stronger suprathreshold responses may be evoked in LMAN neurons of adult sequentially tutored birds by those tutor songs or juvenile versions of the BOS acoustically more similar to the BOS_{Adult}. In this case, the response strength of LMAN neurons to a given song should correlate with the acoustic similarity between the BOS and that song. To investigate this possibility,
we plotted the response strength of individual LMAN neurons to TS1, TS2 or BOSPHD60 as a function of the percent similarity of the BOS to the given song, as measured with either Sound Analysis or subjective methods (Fig. 10). These plots revealed a significant correlation between acoustic similarities and response strength for TS2 and BOSPHD60 but not for TS1 (Fig. 10). In the case of TS1, the absence of a correlation could be accounted for by the fact that only a small fraction of LMAN neurons responded to this stimulus. However, we also noted that the acoustic similarities between the BOSAdult and TS1 estimated using Sound Analysis spanned a range greater than that for the similarities between the BOSAdult and the BOSPHD60 (compare top and bottom left, Fig. 10), suggesting that the lack of responses to TS1 were not simply due to acoustic dissimilarities between the BOSAdult and TS1. Therefore, acoustic similarity as measured with these two techniques can account for LMAN responsiveness only for the most recent tutor song and juvenile versions of the BOS, but not for a tutor song copied only transiently earlier in development.

**Selectivity of LMAN neurons in adult sequentially tutored birds**

Song selectivity is one hallmark of song-evoked responses in the LMAN of normally tutored adult zebra finches, wherein responses are biased to forward playback of the BOS over CON, and to forward over reverse playback of either the BOS or the tutor song from which it was copied (Doupe 1997; Doupe and Konishi 1991). We were curious to know whether LMAN neurons in sequentially tutored adult birds exhibited auditory response properties like those in normal birds, or were more
broadly responsive to songs other than the BOS\textsubscript{Adult} because of their experience with multiple tutors. Therefore, we plotted cumulative distributions of suprathreshold $d'$ values for all 84 song-responsive LMAN neurons that we recorded from in sequentially tutored adult zebra finches, comparing individual neuronal responses to the BOS\textsubscript{Adult} versus either TS1, TS2, BOSP\textsubscript{HD60}, or CON (Fig. 11A). These cumulative distributions indicated that the majority of LMAN neurons were strongly selective for the BOS\textsubscript{Adult} over all of the other song stimuli (i.e., a majority of cells showed $d' > 0.5$). Furthermore, the $d'$ distribution for the BOS\textsubscript{Adult} versus TS1 completely overlapped the distribution for BOS\textsubscript{Adult} versus CON ($p = 0.94$, K-S test), while the distribution for the BOS\textsubscript{Adult} versus TS2 comparison was shifted significantly leftwards (i.e., in the direction of decreased selectivity for BOS\textsubscript{Adult}). The mean $d'$ values revealed equivalently strong selectivity for BOS\textsubscript{Adult} over TS1, BOSP\textsubscript{HD60}, or CON, while the mean selectivity for BOS\textsubscript{Adult} versus TS2 was significantly lower than these other values (Fig. 11B; see Figure legend for statistical comparisons). In addition, LMAN neurons were strongly selective for the forward over reverse playback of BOS\textsubscript{Adult} ($d'_{\text{BOSAdult -- REV:}} 1.45 \pm 0.14$, $n = 27$), and the mean $d'$ value for this comparison was almost identical to that obtained from a large sample of LMAN neurons recorded intracellularly in normal adult zebra finches ($d'_{\text{BOSAdult -- REV:}} 1.41 \pm 0.08$; $n = 235$ cells; (Rosen and Mooney 2000)). The subset of LMAN neurons responsive to TS2 were also selective for forward over reverse playback of this song ($d'_{\text{TS2 -- TS2REV:}} 1.51 \pm 0.14$, $n = 22$ cells). In summary, LMAN neurons in sequentially tutored adult zebra finches are highly selective for the BOS\textsubscript{Adult} over other song stimuli, with diminished selectivity for BOS\textsubscript{Adult} versus the second of the two tutor songs to which the bird was
exposed during development.

**Developmental changes in LMAN auditory responses**

To summarize the developmental changes in LMAN auditory responses in sequentially tutored birds, we plotted the mean response strengths and selectivity for neurons recorded in juvenile and adult birds (Fig. 12). This comparison revealed that LMAN neurons in adult birds lost responsiveness and selectivity for juvenile versions of the BOS and also lost responsiveness to the tutor model on which these juvenile songs were partly based. In particular, the effectiveness of the BOS\textsubscript{PHD60} and TS1 at driving suprathreshold responses diminished between PHD60 and 115 (Fig. 12).
DISCUSSION

We found that sequential tutoring results in serial learning from two different tutors, and LMAN neurons in serial learners respond to the current BOS and the tutor song from which the BOS is copied, but not to prior models or their transient copies. Thus, juvenile finches display behavioral flexibility in learning from multiple models and contain LMAN neurons that are highly plastic in their ability to retune as the juvenile imitates new models. Furthermore, transiently produced songs and their models evoke auditory responses in the LMAN of the juvenile, but these responses are either overwritten or lost in the adult. These results suggest that song-evoked responses of LMAN neurons are the auditory representation of the animal’s current vocal motor program, rather than auditory memories of all songs learned by the individual during its song development.

Behavior of sequentially tutored birds

The sequential tutoring approach provided juveniles with two tutors, affording them an opportunity to copy two song models. Birds tutored in this manner could copy from each tutor, overwriting the copy of the first model as the bird mimicked the second tutor. Just prior to the introduction of the second tutor, the BOS exhibited features indicative of partial copying from TS1. This early copying was most apparent in juvenile zebra finches first exposed to a Bengalese finch tutor, but could also be seen in some juvenile birds raised initially by another conspecific. In contrast, sequentially tutored adults produced songs with copied syllables derived largely from the second and not the first tutor, although more subtle effects of TS1 may have persisted in the adult song of some birds, as
indicated by the elevation in Sound Analysis similarity scores >50%. Certain songbird species, including swamp and song sparrows, copy from several different tutors, then undergo selective attrition, retaining only a subset of these vocalizations to form a more limited suite of adult song types (Marler and Peters 1982a; Marler and Peters 1981; Nordby et al. 2000). In their capacity to mimic multiple vocal models and retain only a subset of these juvenile imitations in the adult vocal repertoire, sequentially tutored zebra finches roughly resemble these other songbirds. One issue emerging from our results is whether the serial “bilingual” birds we studied retain permanent auditory or motor memories of the songs they transiently learn. In swamp sparrows, song material deleted in the first year can be re-expressed during periods of plastic song production in subsequent springs, implying retention of auditory or motor memories of the “lost” songs (Marler and Peters 1982b). If such auditory memories persist, then one may wonder whether early experience, especially of other species’ songs, extends perceptual performance, as for humans with early experience of a language distinct from their native tongue (Tees and Werker 1984).

Sequential tutoring may more closely approximate some aspects of zebra finch song learning in the wild than protocols using only a single tutor. Juvenile zebra finches become independent ~ PHD 35 (i.e., slightly after exposure to TS1 in these experiments), potentially exposing them to the songs of other conspecifics in addition to their father’s song (Immelmann 1969; Zann 1996). Social interactions are especially important in determining which of several potential tutor songs a juvenile subsequently copies, including feeding by adult males (Immelmann 1969) and tutor aggression (Clayton 1987). The
importance of extended social interactions increases the likelihood that birds other than the father will serve as the tutor. Indeed, songs experienced later in the sensory acquisition phase (i.e., PHD 35-60) seem to be especially important in shaping the final BOS: juvenile zebra finches sequentially tutored without an intervening isolation period and introduced to the second tutor at PHD 35 develop adult songs copied predominantly from this second tutor (Eales 1985). Although juvenile copying from the first tutor was not addressed in this earlier study, we report here that early models are imitated transiently and that adult sequentially tutored birds copied almost exclusively from the second tutor, rather than fusing material from the two sources. The tendency to learn material from a single tutor may be a zebra finch specialization (Bohner 1983; Clayton 1987), and clearly differs from songbirds with multiple song types.

Although isolation extended the bird’s capacity to memorize a new model beyond PHD 60, it did not delay song stabilization, which was achieved by ~ PHD 100, when zebra finches normally crystallize their songs (Immelm ann 1969; Zann 1996). This pattern of condensed motor learning resembles other delayed learning protocols (Morrison and Nottebohm 1993; Tchernichovski et al. 2001). Because some studies suggest that crystallization is chronologically invariant (Funabiki and Konishi 2003; Pytte and Suthers 2000), more complete copying might have occurred following an earlier introduction of the second tutor, as in other sequential tutoring studies (Eales 1985).

**Electrophysiological correlates of sequential learning**

Given the capacity for serial copying, one possibility is that areas important to song learning
retain vestiges of early auditory/vocal experience, even when this experience differs from the current song. In other systems, including visually calibrated auditory space maps in owl midbrain (Feldman and Knudsen 1997) and areas of binocular convergence in the frog optic tectum (Udin 1983), distinct synaptic projections patterned by conflicting types of juvenile experience are maintained into adulthood. One area of the songbird brain influenced strongly by experience of the BOS and the TS is LMAN: developmental changes in structure and function of synapses within LMAN are retarded in birds raised without a tutor (Livingston et al. 2000; Wallhausser-Franke et al. 1995), and LMAN neurons of late juvenile (i.e., ~ PHD 60) and adult zebra finches are strikingly selective to playback of the BOS (Doupe and Konishi 1991; Solis and Doupe 1997). These features make LMAN a likely site to search for persistent changes wrought by early song learning experience.

Consistent with the behavioral evidence of transient copying of TS1, the initial song learning experience clearly influenced juvenile LMAN response properties. We found that LMAN neurons in juvenile birds subjected to only the first tutoring and isolation phases responded strongly to the juvenile BOS and to TS1. Rather than reflecting properties of poorly tuned neurons, these responses were highly selective for playback of the juvenile BOS and the TS1 over either of these songs played in reverse, and for the BOS versus other conspecific songs. Therefore, even at a stage of development where copying was rudimentary, and despite a prolonged period of isolation from the tutor, LMAN neurons displayed selective responses to both the BOS and TS, resembling LMAN neurons in PHD 60 birds reared with unrestricted tutor access (Doupe and Konishi 1991; Solis and Doupe 1997).
Therefore, early experience of the juvenile BOS and/or TS1 influences auditory properties of LMAN neurons, raising the possibility that such effects persist in the adult LMAN.

In contrast to this idea, LMAN neurons in the adult do not maintain responsiveness to songs that the bird transiently produced or to the tutor songs upon which these ephemeral vocalizations are based. Despite the development of selective BOS- and TS1-evoked responses in the juvenile LMAN, no sub- or suprathreshold auditory responses were evoked in LMAN by TS1 or BOS\textsubscript{PHD60} in those adult birds that had copied previously from the first of their two tutors. Although a small fraction of LMAN neurons in all of the adult birds we recorded from did respond selectively to TS1 in the adult, these neurons were from birds exhibiting no clear evidence of early copying based on subjective analysis. Throughout development, the response profiles of LMAN neurons shift to the current song (and the most recent TS on which it is based), but highly selective responses for the juvenile BOS are not maintained in the adult. Together with the electrophysiological data in juveniles, these results indicate that selective responses to the current BOS and the first tutor are present at PHD 60, but are lost or overwritten as the bird copies from the second tutor. Therefore, auditory tuning in LMAN differs from sensory tuning in the barn owl and the frog, where two conflicting sensory representations formed during early development are maintained in the adult brain, albeit with one representation persisting as latent synaptic connections (Brickley et al. 1994; Zheng and Knudsen 1999).

Unlike neurons in primary sensory areas, LMAN neurons exhibit song-related motor and auditory activity (Hessler and Doupe 1999). Therefore, LMAN tuning could be influenced by auditory...
experience, by song motor activity, or by combinations of factors, including auditory experience of one’s own song in the context of either the memorized model or song motor activity. The most compelling evidence that the BOS evokes LMAN activity in the context of the memorized model is that LMAN neurons in adult birds with songs disrupted developmentally by tracheosyringeal nerve section are largely unresponsive to BOS playback (Solis and Doupe 2000). In this study, acoustic similarity to the BOS may account for TS2-but not TS1-evoked responses in LMAN neurons of the adult (see Figure 10). The findings reported here indicate that auditory and/or motor experience of the BOS in the context of the current tutor model influences LMAN auditory tuning, consistent with prior evidence that self-generated vocalizations are the key determinant of auditory selectivity in the song system (Doupe 1997; Volman 1993). One prediction of a model where self-generated experience dictates LMAN responsiveness is that tracheosyringeal nerve section in the adult will induce LMAN neuronal retuning. Finally, if songbirds that learn transiently retain “discarded” auditory or vocal motor memories, rather than losing or overwriting them with their current song, these memories are stored in other areas of the songbird’s brain or in forms other than BOS-selective neurons.
FIGURE LEGENDS

**Figure 1.** Zebra finch song development, sequential tutoring and the LMAN recording methods used in this study.

A) In normal zebra finch song development, males memorize a tutor song model during a sensory acquisition phase between posthatch days (PHD) 20 and 60, and vocally copy this model during a sensorimotor learning phase, PHD 40 - 90. Around PHD 90 the plastic song of the juvenile zebra finch becomes acoustically stable, a process referred to as “crystallization.” In the sequential tutoring protocol used here, juvenile male zebra finches were exposed to the song of the first tutor (TS1) until PHD 30 and then isolated for 30 days, then exposed to the song of a second tutor (TS2) for 30 days, followed by another isolation period. Songs were recorded just before and during the exposure to the second tutor, and also after the end of the second isolation period (> PHD 115).

B) Intracellular recordings were made from LMAN neurons and free field song playback was used to measure their auditory responses. LMAN neurons from urethane-anesthetized adult birds (> 115 PHD) were tested with various song stimuli, including the bird’s own song (BOS\textsubscript{Adult}), the BOS produced at PHD 60 (just prior to introduction of the second tutor; BOS\textsubscript{PHD60}), the two tutor songs (TS1 and TS2), these various songs played in reverse (Song-REV), and other zebra finch songs (CON). Recordings from LMAN neurons in another set of juvenile birds were made at the end of the first isolation period (PHD 60). Song stimuli used for these birds included BOS\textsubscript{PHD60}, TS1, REV and CON. Current clamp records (shown as trace to the right of the recording electrode) were used to assess subthreshold and
action potential responses. The connectivity of LMAN is shown in the flowchart within the balloon; the nuclei HVC, RA and the VRN (brainstem vocal respiratory network) are obligatory for singing and form a descending pathway that controls muscles of the avian organ of song, known as the syrinx. LMAN is part of an anterior forebrain pathway that is essential to audition-dependent vocal plasticity, and receives indirect auditory input from HVC, through intervening structures including a basal ganglia homologue area X and the thalamic nucleus DLM.

**Figure 2.** Songs recorded immediately prior to introduction of the second tutor showed hints of copying from the first tutor.

A) Sonograms, generated using Sound Analysis software, depict BOSPHD60 (recorded immediately before exposure to the second tutor) from two different juveniles (Bird 1 and Bird 2) and their first tutor’s song (TS1; another zebra finch). Some hints of copying from TS1 can be seen in the songs of both juveniles. Syllables in the given BOS that are copied from the tutor song are denoted by the same letter used for the given tutor syllable, and marked by an apostrophe (d’ in Bird 2 is copied from d in TS1). The song of Bird 1 showed 50.2% and 25% similarity to TS1 by Sound Analysis (SA) and subjective scoring, respectively, while the song of Bird2 showed 58.3% and 17% similarity to TS1 using these two methods of analysis.

B) Song similarity between BOSPHD60 and TS1, as measured using SA and by subjective scoring, in all 15 birds in which first tutor was another zebra finch. With subjective scoring, 3 of 15 birds were
deemed to have some similarity to TS1, whereas no similarity was detected between BOS_{PHD60} and the second tutor’s song (unrelated birds at 60 PHD, data not shown). Numbers in parentheses denote number of songs with identical scores.

C) Mean similarity scores comparing BOS_{PHD60} to TS1 or CON using either Sound Analysis or subjective scoring, for the same birds as in B). The Sound Analysis comparison used four different CON songs, while the subjective scoring comparison used a single CON song (TS2, to which the bird had not been exposed). The mean similarity of BOS_{PHD60} to TS1 was not significantly higher than that to any of four CON songs, based on an ANOVA.

Figure 3. Sequential tutoring with Bengalese and then zebra finches revealed sequential copying from the first and second tutor.

A) Sonograms of a Bengalese finch TS1 (top), the song of the juvenile reared with this tutor (BOS_{PHD60}; second from top), the TS2 that this juvenile was exposed to starting at PHD 60 (i.e., another zebra finch; third from top) and the final song of this sequentially tutored “BZ” bird (BOS_{Adult}; bottom). Bengalese finch song is distinguished from zebra finch song by relatively shorter duration syllables that are often repeated, features mimicked in the BOS_{PHD60}. After being tutored by a zebra finch (TS2), the song lost these qualities and became zebra finch-like, characterized by long syllables and a defined motif. Copied syllables from either tutor are denoted in the BOS by the same letter used for the given tutor syllable, and marked by an apostrophe. This bird’s song showed 71.8% (Sound Analysis) and 60%
(subjective scoring) similarity to TS1 and 33.4% and 0% to TS2 at 60 days. At 120 days, his song (BOS\textsubscript{Adult}) showed 25.1% and 0% similarity to TS1 and 75.1 and 86% similarity to TS2, using Sound Analysis or subjective scoring methods.

B) Individual similarity scores of BOS\textsubscript{PHD60} and TS1 measured using Sound analysis and subjective scoring (numbers in parentheses in left graph denote number of songs with identical scores).

C) Mean similarity scores using these two measures for BOS\textsubscript{PHD60} and TS1 or CON (right), for juvenile zebra finches initially tutored by a Bengalese finch (n = 11). In the Sound Analysis comparison, BOS\textsubscript{PHD60} was more similar to TS1 than to CON songs, as determined by an ANOVA. In the subjective scoring comparison, the BOS\textsubscript{PHD60} was more similar to TS1 than to CON (i.e., TS2; paired t-test, p < 0.01).

**Figure 4.** Copied syllables in the adult song of sequentially tutored birds were from the most recent tutor (TS2), and not from TS1.

A) Examples of songs from a sequentially tutored zebra finch and his 2 zebra finch tutors. The sonograms depict the BOS\textsubscript{PHD60}, the BOS\textsubscript{Adult}, the TS1 and the TS2. Syllables in the BOS\textsubscript{Adult} that were copied from TS2, as determined by subjective scoring, were labeled with same letter marked by an apostrophe. In this example, the majority of subjective scorers detected no similarity between the BOS\textsubscript{PHD60} and TS1. The BOS\textsubscript{Adult} showed 44.9% and 0% similarity to TS1 based on Sound Analysis and subjective scoring, respectively, and 62% and 87% (6 of 7 syllables copied) similarity to TS2 by
Sound analysis and subjective scoring.

B) Song similarity scores between the BOS_{Adult} of 22 sequentially tutored adult birds and their 2 tutors (TS1 and TS2) measured by Sound Analysis (*left*) or by observers that were blind to the tutoring sequence (*right*; for details see Methods). The songs of 15/20 birds were more similar to TS2 than TS1 based on similarity scores using Sound Analysis. Subjective scores indicated that the BOS_{Adult} showed similarity to TS2 in 17 birds, while failing to find similarity to TS1 in 18/22 birds. Filled symbols represent birds that were sequentially tutored by two zebra finches ("ZZ" birds); open symbols represent birds that were tutored initially by a Bengalese finch and then by a zebra finch ("BZ" birds). Numbers in parentheses denote number of songs with identical scores.

Figure 5. The BOS changes during and after exposure to the second tutor, becoming more like the second tutor’s song.

A) Difference scores show that the songs of most sequentially tutored birds became more like TS2 between PHD 60 and 115, whereas similarity of most of these songs to TS1 either decreased or stayed the same. Difference scores for each bird were calculated by subtracting the similarity score of the BOS_{PHD60} to a given tutor song from the similarity score of the BOS_{Adult} and that same tutor song; positive values indicate increased similarity over development. Differences in similarity were calculated for Sound Analysis (*left*) and subjective scoring (*right*). ZZ and BZ birds are marked by open circles and filled stars, respectively. Numbers in parentheses denote number of scores.
B) The timing of the changes to the BOS and the onset of song stabilization in sequentially tutored birds.

Sound Analysis was used to estimate the similarity of the BOS recorded before, during and after the second tutoring period to the BOS_{Adult}. The BOS started to change soon after introduction of the second tutor, and was not significantly different from the BOS_{Adult} within two months after beginning of second tutor period (time points where the similarity of the current BOS is significantly different from the final BOS are marked by asterisks; number of birds recorded at each time is given in parentheses). The approximate extent of second tutoring period is depicted by the crosshatched box.

**Figure 6.** LMAN neurons in juvenile (PHD60) birds are strongly and selectively responsive to the current BOS (BOS_{PHD60}) and also respond to the tutor song (TS1) heard during the first month posthatch.

A) An example of song-evoked responses from an LMAN neuron in a PHD60 juvenile bird that had been tutored with a zebra finch for the first posthatch month, then isolated for another month prior to the electrophysiological recording. Both the bird’s own song (BOS_{PHD60}) and tutor song (TS1) evoked strong subthreshold (averaged membrane potential records; *middle*) and suprathreshold (cumulative PSTH; *top*) responses, whereas another zebra finch song (CON) that the juvenile bird had not been exposed to previously failed to evoke a response. Response strength (RS; spikes/sec) and p values for the suprathreshold responses are shown above the PSTH for each stimulus. PSTH bin size is 25msec; data are from 30 iterations of each song stimulus, shown as an oscillogram at the bottom.
B) Mean response strength values (RS; spikes/sec) for the various song stimuli for the population of LMAN neurons in PHD 60 birds that responded to at least one of the test stimuli (n = 37 cells). The BOS_{PHD60} evoked the strongest response in the population of LMAN neurons tested here (asterisk indicates $p < 0.05$, ANOVA test).

C) Mean subthreshold responses (Zarea) for various song stimuli for the population of LMAN neurons in PHD 60 birds that responded to at least one of the test stimuli (n = 35 cells). As with the suprathreshold responses, the BOS_{PHD60} evoked the strongest subthreshold responses in the population of LMAN neurons recorded in PHD 60 birds (asterisk indicates $p < 0.05$, ANOVA test).

D) The similarity of the BOS_{PHD60} to TS1 as measured with subjective scoring did not correlate with the TS1-evoked response strength (RS; spikes/sec) in PHD 60 birds. This lack of correlation differed from that seen in adult birds with respect to the acoustic similarity and responsiveness to TS2 (see Fig. 10). The responses of neurons recorded from birds exposed to zebra finch tutors are marked by filled squares (ZF), and those from birds initially tutored by a Bengalese finch (BF) are depicted with open circles.

E) Mean d’ values indicate that LMAN neurons in juvenile birds are selective ($d’ > 0.5$) for the BOS_{PHD60} versus either CON or TS1 (left), and are also selective for forward versus reverse playback of either the BOS_{PHD60} or TS1 (right). The mean d’ value for BOS_{PHD60} versus TS1 was not significantly lower than the d’ value for BOS_{PHD60} versus CON, as determined by a Student’s t-test.

F) The cumulative distribution of d’ values comparing responses evoked by the BOS_{PHD60} with those evoked by TS1, CON or BOS_{PHD60} REV (REV) are largely overlapping, indicating equivalent selectivity
for the BOS\textsubscript{PHD60} over the other stimuli.

**Figure 7.** In adult sequentially tutored birds, LMAN neurons responded to playback of the current BOS and the most recent tutor song, but not to earlier versions of the BOS or earlier tutor songs. A representative LMAN neuron from an adult sequentially tutored bird responded to the BOS\textsubscript{Adult} and TS2, but not to TS1 or the BOS\textsubscript{PHD60}, at both the subthreshold (average membrane potential records; *middle*) and suprathreshold level (cumulative PSTHs; *top*). Response strength (RS; spikes/sec) and p-values, from a paired t-test comparing the stimulus-evoked and baseline firing rates on a trial-by-trial basis, are given above the PSTH for each stimulus condition. PSTH bin size is 25 msec; data are from 21 iterations of song playback; song stimuli are shown as oscillograms at bottom.

**Figure 8.** Average response strengths of LMAN neurons in adult sequentially tutored birds to juvenile and adult versions of the BOS, as well as to the two tutor songs experienced by the bird. A) The average response strength (RS; spikes/sec) from the 84 responsive LMAN neurons we recorded in adult birds, showing that the BOS\textsubscript{Adult} is the most effective stimulus, followed by TS2, BOS\textsubscript{PHD60}, CON and TS1; reverse playback of either the BOS\textsubscript{Adult} or TS2 (BOS\textsubscript{Adult} REV and TS2 REV) did not drive responses in these LMAN neurons. At a population level, the BOS\textsubscript{Adult} elicited significantly stronger responses than any other song, while TS2 evoked greater mean responses than all other songs (asterisk indicates p <0.05, ANOVA test). Data are from 21 animals, including 15 ZZ and 6 BZ birds.
B) Mean RS values in LMAN neurons in those seven adult birds that had clearly copied from their first tutor (TS1) at PHD60 (i.e., just prior to exposure to the second tutor), as determined by subjective scoring (four of these seven birds were initially tutored by a Bengalese finch). In this subset of cells (n = 29), the mean suprathreshold response of LMAN neurons was greatest for the BOS\textsubscript{Adult} and for TS2 (asterisk indicates p < 0.05, ANOVA test). None of these LMAN neurons (0/29 cells) responded to TS1.

Figure 9. Average subthreshold responses of LMAN neurons in adult sequentially tutored birds to juvenile and adult versions of the BOS, as well as to the two tutor songs experienced by the bird.

A) The average z-scores for positive area (Zscore\textsuperscript{+area}; see Methods) from the 68 responsive LMAN neurons we recorded in adult birds, showing that the BOS\textsubscript{Adult} is the most effective stimulus, followed by TS2, BOS\textsubscript{PHD60}, CON and TS1; BOS\textsubscript{Adult-REV} and TS2-REV did not drive responses in these LMAN neurons. For CON, REV and TS2-REV we tested a subset of 68 neurons, 27, 21 and 21 respectively. At a population level, the BOS\textsubscript{Adult} elicited significantly stronger responses than any other song, while TS2 evoked greater mean responses than all other songs (asterisk indicates p < 0.05, ANOVA test). Data are from 21 animals, including 15 ZZ and 6 BZ birds.

B) Mean subthreshold responses (Zscore\textsuperscript{+area}) in LMAN neurons in those seven adult birds that had clearly copied from their first tutor (TS1) at PHD60 (i.e., just prior to exposure to the second tutor), as determined by subjective scoring (n = 3ZZ and 4BZ birds). In this subset of cells (n = 24), the mean
subthreshold responses of LMAN neurons were greatest for the BOS\textsubscript{Adult}, and greater for TS2 than for BOS\textsubscript{PHD60}, BOS\textsubscript{Adult} REV and TS2 REV (asterisk indicates p < 0.05, ANOVA test). In contrast, none of these LMAN neurons (0/24 cells) responded to TS1.

C) Subthreshold peak (P) analysis of LMAN neurons in adult sequentially tutored birds detected two more TS1-responsive cells, and a much larger number (22) of TS2-responsive cells, as contrasted with a whole motif analysis (W). These results suggest that latent responses to TS1 are not present in the LMAN of adult birds. For peak analysis, we measured the subthreshold response measured during a short time window (~ 200 - 800 msec; see Methods) bracketing the peak response evoked by the tutor’s song.

Figure 10. The response strength to either the juvenile BOS or the first or second tutor song plotted as a function of the acoustic similarity of that song to the adult BOS. The relationship of response strength (RS; spikes/sec) to each of the two tutor songs that the bird was exposed to during development (TS1 (top) and TS2 (middle)) and old versions of own song (BOS\textsubscript{PHD60}; bottom), plotted as a function of the similarity of that tutor song to the BOS\textsubscript{Adult}, calculated using either Sound Analysis (left column) or subjective scoring methods (right column). A significant correlation was detected between the response strength to TS2 or BOS\textsubscript{PHD60} and the similarity of either of these songs to the BOS\textsubscript{Adult} as assessed with either analysis method (middle and bottom). In contrast, no significant correlation was detected between the response strength to TS1 and the acoustic similarity of TS1 to the BOS\textsubscript{Adult} (top).
LMAN neurons from ZZ birds are plotted as filled squares, while those from BZ birds are plotted as open circles.

**Figure 11.** The auditory responses of LMAN neurons in adult sequentially tutored birds are strongly selective for the BOS$_{\text{Adult}}$ over other songs.

A) A cumulative distribution of suprathreshold d’ values (see Methods) for the BOS$_{\text{Adult}}$ versus other songs used as test stimuli, including BOSPHD60, TS1, TS2, CON and BOS$_{\text{Adult-REV}}$. Distributions were similar for all songs, although the distribution for the BOS$_{\text{Adult}}$ versus TS2 was shifted leftwards (in the direction of decreased selectivity; BOS$_{\text{Adult}}$ -TS2 versus: BOS$_{\text{Adult}}$ -TS1, p < 0.001; BOS$_{\text{Adult}}$ -BOSPHD60 p = 0.01; BOS$_{\text{Adult}}$ -CON; p = 0.001, K-S tests). Cells with responses biased to the BOS$_{\text{Adult}}$ exhibit d’ > 0.5, whereas d’ < -0.5 denotes a response bias to the other song. Values between –0.5 and 0.5 (shaded region) indicate that the neuron shows no net bias to either of the stimuli in the pair.

B) LMAN neurons in sequentially tutored adult birds exhibited heightened selectivity for the BOS$_{\text{Adult}}$ versus other stimuli. The mean d’ values for all responsive neurons (n = 84) were similar for the BOS$_{\text{Adult}}$ versus TS1, BOS$_{\text{Adult}}$ and CON, but significantly less for BOS$_{\text{Adult}}$ –TS2 (asterisk indicates p < 0.05, ANOVA test).

**Figure 12.** A developmental comparison of LMAN auditory responses in juvenile and adult sequentially tutored birds reveals loss of responsiveness for early generated songs and a loss of
responsiveness to the tutor model on which these juvenile songs are based. The mean response strength (RS; spikes/sec) to a given song at PHD60 prior to exposure to the second tutor (open circles) and in adults following exposure to the second tutor (filled squares). Significant decreases in responsiveness to the BOS \textsuperscript{PHD60} and TS1 occurred over development. Also note the equivalent RS values for the BOS and the most recent tutor song at the two developmental time points. “REV” refers to reverse playback of the relevant (i.e., age-matched) BOS. \(P\) values obtained using a Welch’s t-test (BOS \textsuperscript{PHD60}) and Student’s t-test (TS1).
Acknowledgements:

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Table 1 Song learning in sequentially tutored birds

### Recorded at adult stage of development

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<tr>
<th>Tutor(s)</th>
<th>bird #</th>
<th>from TS1</th>
<th>range</th>
<th>mean±SE</th>
<th>from TS2</th>
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<td>0 - 1</td>
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<td>2 - 9</td>
<td>4.5 ± 0.42</td>
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### All birds at PHD 60 (includes birds not used for electrophysiology)

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<td>B</td>
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#### Duration of syllables (ms)

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<td>B</td>
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Table 2 LMAN song-evoked responses

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<th># of neurons responsive to given song</th>
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Recorded at juvenile stage of development

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Figure 1  Yazaki-Sugiyama & Mooney
11/2 columns
Figure 2  Yazaki-Sugiyama & Mooney
11/2 columns
Figure 3  Yazaki-Sugiyama & Mooney
2 columns
Figure 4  Yazaki-Sugiyama & Mooney
1 column
Figure 5  Yazaki-Sugiyama & Mooney
11/2 columns
Figure 6  Yazaki-Sugiyama & Mooney
2 columns
Figure 7  Yazaki-Sugiyama & Mooney
11/2 columns
Figure 8  Yazaki-Sugiyama & Mooney
1 column
Figure 9  Yazaki-Sugiyama & Mooney
Figure 10 Yazaki-Sugiyama & Mooney
2 columns
Figure 11  Yazaki-Sugiyama & Mooney
1 column
Figure 12  Yazaki-Sugiyama & Mooney
1 column