# Changes in Adult Zebra Finch Song Require a Forebrain Nucleus That Is Not Necessary for Song Production

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**ABSTRACT:** Male zebra finches normally crystallize song at approximately 90 days and do not show vocal plasticity as adults. However, changes to adult song do occur after unilateral tracheosyringeal (ts) nerve injury, which denervates one side of the vocal organ. We examined the effect of placing bilateral lesions in LMAN (a nucleus required for song development but not for song maintenance in adults) upon the song plasticity that is induced by ts nerve injury in adults. The songs of birds that received bilateral lesions within LMAN followed by right ts nerve injury silenced, on average, 0.25 syllables, and added 0.125 syllables (for an average turnover of 0.375 syllables), and changed neither the frequency with which individual syllables occurred within songs nor the motif types they used most often. In contrast, the songs of birds that received sham lesions followed by ts nerve injury lost, on average, 1.625 syllables, silenced 0.125 syllables, and added 0.75 syllables, turning over an average of 2.5 syllables. They also significantly changed both the frequency with which individual syllables were included in songs and the motif variants used. Thus, song plasticity induced in adult zebra finches with crystallized songs requires the presence of LMAN, a nucleus which had been thought to play a role in vocal production only during song learning. Although the changes to adult songs induced by nerve transection are more limited than those that arise during song development, the same circuitry appears to underlie both types of plasticity. © 1999 John Wiley & Sons, Inc. J Neurobiol 39: 14–28, 1999

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Songbirds learn to sing early in development, matching their own vocal output to a remembered song model, but song plasticity may be limited or absent after sexual maturity. Some species such as the canary may relearn portions of their song each succeeding year (Nottebohm et al., 1986) and so experience multiple sensitive periods resulting in repeated relearning of song, while others such as the zebra finch do not change their songs after reaching sexual maturity, when they crystallize their adult song (Immelmann, 1969; Price, 1979). Among the neural and physiological correlates of the change from plasticity to a crys-tallized stereotyped song in zebra finches are (a) a surge in testosterone levels (Pröve, 1983), which in swamp sparrows causes increased song stereotypy (Marler et al., 1988); (b) myelination of fibers within and connecting song system nuclei in the forebrain (Herrmann and Bischof, 1986); (c) changes in the auditory specificity of neurons within forebrain song nuclei (Volman and Konishi, 1987; Doupe, 1997); and (d) loss of the capacity for lesions placed in the nuclei of the lateral recursive loop (Area X, DLM, and LMAN) to affect song (Bottjer et al., 1984; Sohrabji et al., 1990; Scharff and Nottebohm, 1991). These correlates of song crystallization may be interdependent, as is suggested by the presence of androgen receptors within LMAN (Arnold et al., 1976) and the role of testosterone in regulating myelination within the song system (Guttinger et al., 1993; Stocker et al., 1994). The finding that extending the sensitive period

for song learning in zebra finches by depriving young birds of song tutors (Eales, 1985) also extends the period during which song can be disrupted by LMAN lesions (Morrison and Nottebohm, 1993) suggests that the link between neural changes and the increase in song stereotypy associated with crystallization appears to be causal. These results have generally been interpreted as signifying that the lateral recursive loop has a role in song learning but not in the maintenance or production of adult song.

After zebra finch song crystallizes at approximately 90 days of age, birds recorded over $\geq 1$ years maintain the same stereotyped song (Price, 1979; H. Williams, unpublished data). However, deafening adult males can result in degradation of song elements (Nordeen and Nordeen, 1992). Similarly, disrupting the output of the song system either by injuring one of the nerves that controls the avian vocal organ, the syrinx, which alters the phonology of song syllables (Williams and McKibben, 1992), or by temporarily silencing the vocal organ (Hough and Volman, 1996) leads in some birds to changes in the stereotyped song. These changes result in a reorganization of the song, with some syllables dropped and others added, yielding a new temporal configuration of the song pattern that becomes stable and stereotyped within 3–4 months. Although these changes to the song do not seem to result from imitation of a model, and so cannot be said to be a function of imitative learning, they do represent a marked alteration of the vocal pattern that was learned during development. In this study, we asked whether the alterations to adult song that can be induced by tracheosyringeal nerve injury require the same forebrain song circuitry, the lateral recursive loop, that is necessary for the initial development of song.

## MATERIALS AND METHODS

### Subjects

Adult male zebra finches *Taeniopygia guttata* were bred at Williams College or at the Rockefeller University Field Research Center and caged individually at Williams College in a room at 25°C on a 14:10 h light/dark cycle for the course of the study. The 28 males used ranged from 4 to 60 months of age; all had reached sexual maturity and crystallized their songs (which in zebra finches normally occurs at 3 months) (Immelmann, 1969; Price, 1979). The 28 males were assigned to four groups, in a $2 \times 2$ design with the presence or absence of (a) lesions within LMAN and (b) injury to the tracheosyringeal nerve as the treatments. To ensure that treatment groups were balanced by age, previous history, and song characteristics, assignments to a treatment group were made in two steps: Birds were first matched by

age and song, and then birds within each such matched set were randomly assigned to separate treatment groups. The resulting four groups were: (a) bilateral lesions placed in LMAN and unilateral NXII tracheosyringeal (ts) removal (LMAN/ts, $n = 8$), (b) LMAN lesions alone (LMAN, $n = 6$), (c) sham lesions with unilateral NXIIts removal (sham/ts, $n = 8$), and (d) sham lesions alone (sham, $n = 6$). Each group except the LMAN-lesioned birds included one male that had previously bred.

The average age of the birds in the groups did not differ significantly [$F(3, 24) = 0.59$, $p > .62$], and ranged from $20.0 \pm 6.6$ [mean $\pm$ standard error of the mean (S.E.M.)] months for the LMAN group to $29.9 \pm 7.4$ months for the sham/ts group. All groups included at least one bird 6 months old or younger, and all groups also included at least one bird 3 years old or older. Neither in previous work (Williams and McKibben, 1992) nor in this study was a relationship between age and the extent of adult song changes observed (as tested with a linear model that included treatment group; [$F(1, 3, 24) = 0.02$, $p = .89$]).

Birds were assigned to groups based on song similarities; birds with similar songs were assigned to different groups. The number of syllables in the birds' original songs did not differ among groups [$F(3, 24) = 0.04$, $p > .98$] and ranged from $11.5 \pm 1.13$ (mean $\pm$ S.E.M.) for the LMAN/ts group to $12.0 \pm 1.53$ for the LMAN group. The distribution of syllable types (see below) was also balanced among groups ($\chi^2 = 59.92$, df $= 12$, $p > .75$).

## Song Recording

To record directed (courtship) song, a male bird's cage was placed inside a Lucite chamber ($75 \times 60 \times 50$ cm) containing a microphone and lined on four sides with acoustic foam, and a female was placed immediately outside the chamber in sight of the male. This arrangement allowed us to record courtship song directed at a female without contaminating the recording with the female's calls. Recording directed song is preferred because the motivational state of the male is known and similar for each recording over the course of the study (Sossinka and Böhner, 1980). In preoperative recordings, the birds sang an average of $2.57 \pm 0.36$ introductory notes per strophe, which is consistent with the range for directed song (Sossinka and Böhner, 1980); the treatment groups did not differ in the number of introductory notes sung per strophe [$F(3, 24) = 0.446$, $p > .7$]. The number of songs per strophe ($1.89 \pm 0.11$) in the preoperative recordings was also consistent across treatment groups [$F(3, 24) = 0.431$, $p > .7$].

Songs were either recorded with a dynamic microphone (Marantz EC-7) and a Marantz PMD-201 cassette recorder onto Maxell MS-60 studio tape and then digitized (8 bits at 22 kHz using SoundEdit on a Macintosh Quadra 840AV computer) or recorded digitally (with the same microphone and software directly into a Macintosh 520 computer at 16 bits and 20 kHz). All birds were recorded extensively (several song bouts including at least 30 motifs) within the 3 weeks preceding the initial surgery. After surgery, each bird's song was recorded at 2-week intervals for 14 weeks

following the final surgical treatment and then again at 20, 25, and 30 weeks after the original surgery. Each recording session included either as much song as could be obtained during the course of 1 day or 30 motifs. If males failed to sing during a scheduled session, they were rerecorded until they did sing.

## Song Analysis

Zebra finch song normally consists of a series of distinct and harmonically complex syllables delivered in a stereotyped order, usually but not always separated by intervals of silence. Individual songs, or motifs, are most often 0.5 to 1.5 s long, and several such songs may be sung in sequence to form a strophe. Each strophe is usually preceded by a series of identical short introductory notes. We analyzed several aspects of the song: which syllables were given, the type of the syllables (see below), the order in which they were sung, the spacing and length of the syllables, the tempo of the song, and the fundamental frequency of certain syllables. The scoring of song data used in this report was performed by the first author, who was blind to the LMAN treatment. However, the characteristic appearance of sonograms after right ts nerve injury made it obvious whether a bird had received that treatment.

Song syllables were identified visually from sonograms (generated with SoundEdit). We separated syllables according to the criteria described in Williams et al. (1992), which are consonant with behavioral measures of how birds split song into units. Syllables were defined as those sounds separated from other sounds by short periods of silence or by abrupt transitions in amplitude and/or frequency. This sometimes results in defining more syllables for a given song than do some other methods, but it should also be noted that birds from our colony have longer and more complex songs than do some other colonies of domesticated zebra finches, and in this way they more closely approximate the songs of wild zebra finches (Zann, 1993).

Each syllable was assigned to one of five different types (Williams and McKibben, 1992): stack (an unmodulated or slightly modulated syllable with strong harmonics; 23.3% of syllables in the preoperative songs of birds used in this study); downsweep (syllables with strong harmonics and a fundamental frequency decreasing rapidly during the majority of the syllable; 50.4%); high note (syllables dominated by a single element with a constant frequency above 3 kHz; 10.8%); click train (a series of short rapidly modulated notes; 7.0%); and other (all syllables that did not fall into one of the first four categories; 8.5%).

Following right-side tracheosyringeal nerve injury, the syllable structure of zebra finch songs is disrupted, sometimes severely (Williams et al., 1992). However, it is easily possible to identify syllables by matching the lengths and amplitudes of each sound to the syllables in the preoperative song.

Syllables were scored as lost if they were omitted from the song for at least the two final recording sessions. Syllables were considered to be added to the song if they appeared in a stereotyped form and position in at least one third of the songs recorded in each the final two sessions. Restricting the analysis of song changes to these two measures can underestimate the actual shifts in singing behavior, as when a bird sings nearly all of its motifs as the sequence ABCDEFG before surgery and, though it continues to sings some motifs of the ABCDEFG form after treatment, shifts to singing 90% of its motifs as the sequence ABCD. For this reason, we quantified song changes with two additional measures designed to be sensitive to such alterations in the song: the average change in the frequency with which a syllable is included in the song, and the shift in common motif variants.

To determine the average change in the frequency with which syllables were included in the song, we first determined for each syllable the proportion of preoperative songs that included the syllable. Then, for each subsequent recording we determined the proportion of songs that included the syllable and calculated the absolute value of the difference between the two proportions. For example: syllable B was included in 100% of preoperative songs and syllable G in 25% of those songs, and in the final postoperative recording syllable B occurred in 65% and syllable G in 75% of the songs—the difference was 35% or 0.35 for syllable B and 50% or 0.5 for syllable G. For each recording, the differences for all of the syllables in the song were averaged to obtain an overall measure of the amount of shift in the frequency with which song syllables were included in the song (note that failing to take the absolute value for each individual syllable's change might result in an average value near zero, when in fact large changes in opposite directions might have occurred, as in the example in the previous sentence).

Motif variants were also used for tracking systematic changes in songs. Although a zebra finch song is generally defined as the motif including all of the syllables (for a bird with seven syllables, ABCDEFG), birds often shorten motifs by stopping songs short and occasionally start motifs late (ABCDE or CDEFG). Each distinct subset of song syllables that was sung was termed a motif variant. All songs within a recording were tabulated as to the motif variant they represented, and the frequency of each motif variant was calculated. We then determined the most common motif variants for a recording session as follows: Starting with the motif variant most often sung, we added in turn the variants next most often sung until the list of variants accounted for 80% of the songs sung in the recording session. In most cases, two to four motif variants accounted for 80% of the songs. We were then able to determine shifts in the composition of songs by noting the percentage of the original common motif variants retained as common motif variants in subsequent recordings.

The lengths of songs, syllables, and silences intervening between syllables were measured in five songs in one of the preoperative recordings and five songs in some intermediate recordings as well as in the final song recording (using SoundEdit 16). The five songs included one from the first song bout recorded, one from the last song bout recorded, and three evenly spaced within the recording session; care was taken to use songs with different positions within a bout so that, for example, the first (or last) song in a bout was not

the only song position sampled. Syllables and the silences between them were measured to the nearest 0.1 ms. Songs were measured to the nearest millisecond, and an exactly corresponding portion of the song (whenever possible, the entire song) was measured for each bird's set of recordings. Fundamental frequencies of individual syllables were measured with Signalyze (Infosignal) by measuring values of several harmonics and dividing by the order of the harmonic, a method which allows for greater precision of measurement (see Williams et al., 1992, for full details).

## Surgical Procedures

***Anesthesia.*** Birds were housed without food for 1 h prior to surgery and then injected intramuscularly with 0.05 mL of a 50:50 mixture of ketamine (10 mg/mL) and xylazine (20 mg/mL) supplemented when necessary with inhaled metofane. All birds received injections of yohimbine to reverse the effects of the anesthetic after surgery was completed (Kilander and Williams, 1992).

***LMAN Lesions and Sham Lesions.*** The bird's head was placed in a Kopf stereotaxic apparatus with the beak held at 45° below horizontal using an especially designed bill clamp, and an incision made in the scalp. Holes were made in the skull over LMAN (coordinates: 4.2 mm anterior, 2.0 mm lateral). After an opening was made in the dura, a stainless-steel electrode (00 insect pin coated with Insulex except at the tip) was placed 2.5 mm below the surface of the brain and 90 s of 50 $\mu$A anodal current was delivered to lesion the target nucleus (a silver wire hook in contact with the scalp served as a ground). Because we did not wish the lesions we placed to impinge on song system areas other than lateral MAN (both medial MAN and Area X are nearby), lesion sizes were restricted. After bilateral lesions were placed, the lips of the incision were drawn together and sealed with collodion or acrylic adhesive. For birds that received sham lesions the procedure was identical except that current was not passed through the electrode.

***Tracheosyringeal Nerve Injury.*** The songs of the 16 adult males in the LMAN/ts and sham/ts groups were recorded for 25–50 days after surgery before the right ts nerve was injured. This procedure was used to minimize the possibility that interactions between the two surgeries would produce effects upon song that would be confounded in the analysis. Although long-term effects of LMAN lesions upon neurons in RA and DLM have been described (Johnson and Bottjer, 1993, Akutagawa and Konishi, 1994), these effects occurred during development.

The bird was prepared for surgery and anesthetized, and the head was placed in the stereotaxic unit as described for the LMAN lesions. The bird's body was rotated 180° counterclockwise. An incision was made over the trachea and the right tracheosyringeal nerve was dissected free of the surrounding connective tissue and muscle. The nerve was then cut and the distal stump was pulled out to prevent regeneration (Williams and McKibben, 1992). The right ts nerve was injured because it is more easily accessible and because

injuring the right side has a greater effect upon song, as the zebra finch is right-side dominant for song control (Williams et al., 1992). The incision was sutured and sealed with collodion or acrylic adhesive.

***Histology.*** After the song recording portion of the study was completed, all birds were given an overdose of ketamine–xylazine and perfused with 10% formol saline. At this time, ts nerve stumps were inspected for evidence of regeneration; in all cases, a short (<1-mm) fanlike group of fibers was seen emerging from the stump, but no fibers extended beyond this area (approximately 1 cm from the syrinx).

Brains were sectioned coronally on a Vibratome, and the 50-$\mu$m sections mounted on slides and stained with Cresyl Violet. Alternate sections were digitized (Dage MTI camera and Data Translations board) and the area of LMAN was measured (NIH Image 1.53); the area of LPO (lobus parolfactorius) was also measured for every sixth section for the extent of LMAN. LMAN was defined as including both the oval magnocellular portion of the nucleus and the smaller flattened cells surrounding the area of large neurons in the form of a shell (Johnson et al., 1995). In sections where LMAN and mMAN appeared to be nearly continuous, the narrowest extent of the large cells was taken to be the boundary between the two. The volumes of the left and right LMAN were then calculated and expressed as a proportion of the average area of LPO, to control for shrinkage during processing and for differences in brain size.

To determine the extent of the lesion in cases where LMAN was not completely eliminated, we expressed the LMAN volume as a percentage of the average measurement of total LMAN volume in sham-lesioned birds.

## RESULTS

## Lesions

Of the 14 birds that received lesions, 11 received substantial damage to both LMAN nuclei and all had less LMAN tissue remaining than was present in the smallest LMAN nuclei of the sham-lesioned birds (the extent of the lesions for one bird is shown in Fig. 1). The average volume of LMAN tissue remaining in the lesioned birds was 34% of that in the sham-lesioned birds, and the differences in volume between the two groups were highly statistically significant ($t = 4.831$, $df = 14$, $p = .0003$). Twelve of the 14 lesions included portions of the margin, or shell, of LMAN. None of the lesions impinged upon medial MAN; effects upon LPO were restricted to its dorsal margin and did not involve Area X. As the lesions were electrolytic, fibers of passage may have been affected. Because damage to LMAN was evident in all birds that received lesions, we did not reassign the three birds with relatively small lesions within LMAN to the sham-lesioned groups; this makes our analysis of
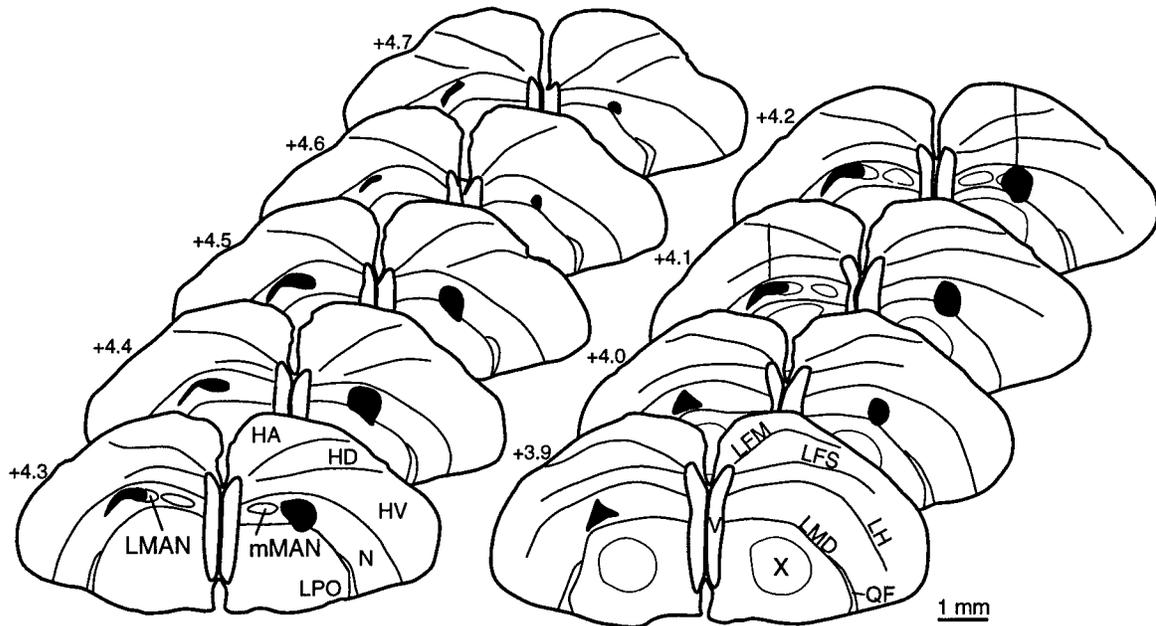
**Figure 1** Lesion placement. Lesions, as defined by histological examination of Cresyl violet–stained sections in bird Bk103, are shown in black. Intact portions of LMAN remained on the medial edge of the nucleus on both sides of the brain; the amount of intact LMAN tissue spared was 16.7% of the average volume of the two nuclei in sham-operated birds. Five birds had more extensive lesions and eight had less extensive lesions than the example shown here. Although the dorsomedial margin of LPO is included in the right side lesion, Area X was not involved. The lesions did not impinge upon medial MAN. Numbers next to each section represent distance (millimeters) anterior to the reference point (the intersection of the cerebral hemispheres and the cerebellum). Abbreviations (after Stokes et al., 1974; Nottebohm et al., 1982): HA = hyperstriatum accessorium; HD = hyperstriatum dorsale; HV = hyperstriatum ventrale; LFM = lamina frontalis suprema; LFS = lamina frontalis superior; LH = lamina hyperstriatica; LMAN = lateral magnocellular nucleus of the anterior neostriatum; LMD = lamina medullaris dorsalis; LPO = lobus parolfactorius; mMAN = medial magnocellular nucleus of the anterior neostriatum; N = neostriatum; QF = tractus quintofrontalis; X = Area X.

behavior in relation to treatment group conservative and thus less likely to show differences between groups.

## Changes in Song Tempo after LMAN Lesions

All of the birds that did not add or lose song syllables increased the average tempo of their songs during the course of the study (although two of the increases were <1%). The tempo of the songs of birds with LMAN lesions was consistent within recording sessions (the average error of measurements within a recording session was 0.43 ms) and increased systematically and steadily between session (average change in tempo = 0.13%/recording). Three of the birds with LMAN lesions increased the tempo of their songs to the extent that songs were sung at least 10% faster at the end of the study than

during the initial recording sessions (Fig 2). The 12 birds with LMAN lesions that did not lose or add syllables to their songs had an average song tempo of 6.875 ± 0.13% faster in their final recordings than they did in the two initial recording sessions, while the eight birds that received sham lesions and did not add or lose song syllables sang their songs an average of 2.5 ± 0.8% faster over the same time period; the difference in increase in tempo approached significance ($t = 1.86$, df = 10, $p = .1$).

The songs of the three birds with LMAN lesions that increased song tempo by more than 10% were further analyzed to determine how the reduction in song length had been achieved. A regression of the average preoperative syllable and silence length against the same unit's final length gave an $r^2 = .96$ ($n = 49$, $p < .0001$), indicating that all temporal units within the song were reduced by the same proportion. However, when individual birds were examined, a
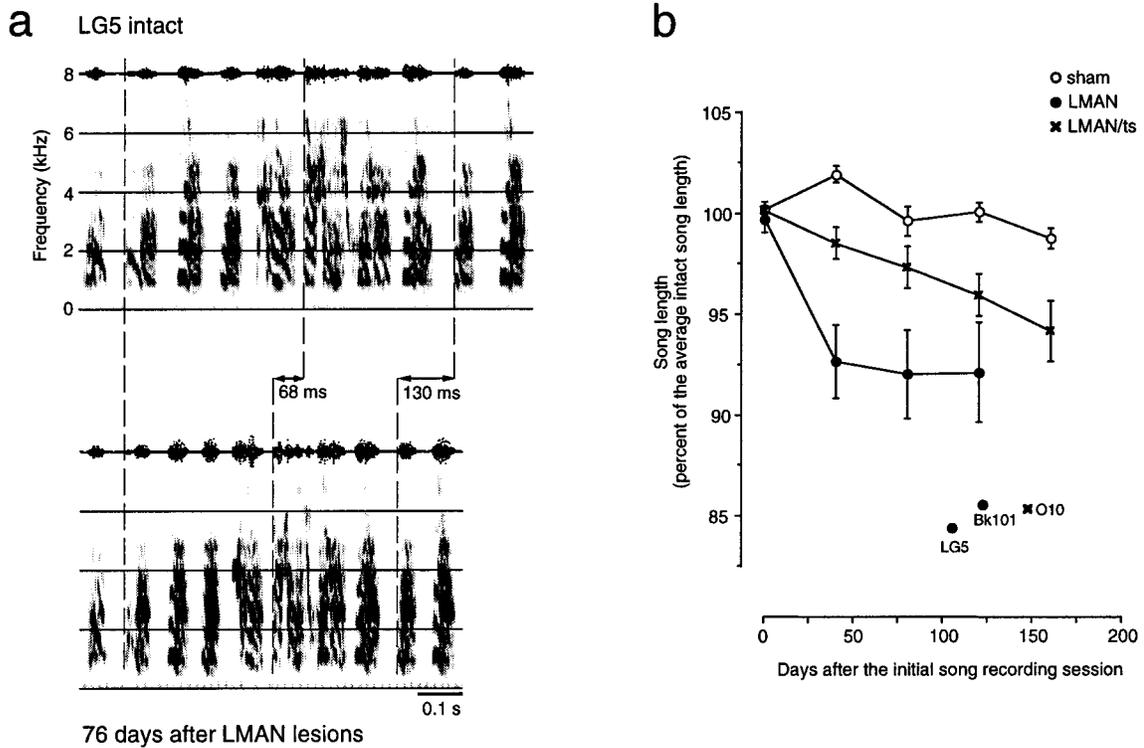
**a** LG5 intact

68 ms    130 ms

0.1 s

76 days after LMAN lesions

**b**

○ sham
● LMAN
✕ LMAN/ts

Bk101    ✕ O10
● LG5

Days after the initial song recording session

Song length (percent of the average intact song length)

**Figure 2** Changes in song tempo. (a) The song of LG5 (including one introductory note) as recorded intact and 76 days after bilateral lesions in LMAN. The song was sung with an increased tempo, resulting in a shorter song, although the frequency of syllables did not change proportionately (compare the short 4-kHz component of the fifth syllable before and after surgery). The reduction in song length occurred linearly throughout the song, as denoted by the constant percentage of the original song length at each point measured within the song. (b) Birds with LMAN lesions showed the greatest overall increases in tempo, birds in the sham group showed no changes, and birds in the in the LMAN/ts group had intermediate increases in tempo (the sham/ts group made substantial changes to their songs, which precluded comparative measures of song length). Song tempo increased over the course of the study, reaching an asymptote at approximately 85% of the original song length in the case of the LMAN group. The three birds that showed the largest changes in song tempo (denoted by labeled points) also had steady changes over time.

slightly different picture emerged: Two of the birds, LG5 and O10, reduced their syllables and silences by the same amounts, while the song of the third bird, Bk101, had shortened syllables but the silences between syllables were preserved at their initial lengths.

To determine whether the birds with large increases in song tempo altered the pitch of their syllables, we measured the fundamental frequencies of three examples of each of two syllables in two of the birds' songs (the third bird had no syllables with regions of constant frequency suitable for measurement). The difference between preoperative and postoperative frequencies was not significant ($t = 1.1$, df $= 11$, $p > .29$). One bird had an average decrease in frequency of $3.1 \pm 1.0\%$, while the other had an average increase in frequency of $2.5 \pm 0.5\%$. Birds with LMAN lesions that increased the tempo of their

songs did so while preserving the fundamental frequencies of the song.

We examined the possibility that damage to LMAN was related to the degree of change in tempo. However, the size of the lesion (as measured by the amount of identifiable LMAN tissue remaining) was not significantly correlated to the change in song tempo ($r = .295$, $n = 14$, $p = .3$).

Directed song is sung at a faster tempo than undirected song (Sossinka and Böhner, 1980), and it is possible that the birds that sang with increased tempo did so because they sang more directed songs. However, two measures of song that differentiate between directed and undirected song, the number of introductory notes per strophe and the number of motifs per strophe, were not significantly correlated with the changes in tempo in birds that received LMAN le-

sions ($r = .148$, $n = 14$, $p = .61$ for introductory notes per strophe; $r = .337$, $n = 14$, $p = .24$ for motifs per strophe). Given these observations, it is unlikely that the increases in tempo we observed were due to a change in the proportion of directed song.

## LMAN Lesions Did Not Affect Song Syllables or Stereotypy

None of the 12 male zebra finches that received LMAN lesions or sham lesions but not ts nerve injuries added or lost syllables from their songs, nor did the LMAN- and sham-lesioned birds differ on any other measure of song change. On average, the frequency with which a syllable was sung changed by $7.5 \pm 1.4\%$ for LMAN-lesioned birds and by $7.0 \pm 2.3\%$ for sham-lesioned birds between the first and the last recordings, indicating that only small shifts in the composition of songs occurred. Comparing the two groups showed that in the absence of ts nerve injury, lesions placed in LMAN had no significant effect upon average shift of song syllable inclusion within songs ($t = 0.187$, df $= 10$, $p > .85$).

Likewise, there was no substantial shift in the motif variants that birds without ts nerve injuries lesions used most often within their songs. Among the sham-lesioned birds, $95.8 \pm 4.2\%$ of the most common preoperative motif variants were retained among the most common motif variants at the end of the study. Although the LMAN-lesioned birds retained fewer of the most common preoperative motif variants at the end of the study, $82.0 \pm 5.9\%$, the difference did not reach significance ($t = 1.932$, df $= 10$, $p = .08$).

## Changes in Songs after ts Nerve Injury to LMAN- and Sham-Lesioned Birds

Tracheosyringeal nerve injuries induced changes in the songs of birds in both the sham- and LMAN-lesioned groups. However, the sham-lesioned birds made substantially more changes to their songs in both number and type. Both groups included birds that silenced song syllables, but only the sham-lesioned birds added and deleted song syllables, and the changes in motif variants and in the average shift in frequency with which syllables were included within songs were greater for the sham/ts birds than for the LMAN/ts birds.

*Syllables Added and Lost.*  Birds in the sham/ts group added six syllables to their songs (four birds) and lost 14 syllables (four birds); all but two (LB76 and Pk28) of the eight birds added and/or lost syllables (Table 1, Figure 3). Birds in the LMAN/ts group added one

syllable to their songs, while four syllables were lost: one from each of two birds and two from a third bird. The LMAN/ts bird that lost two syllables, Bk66, lost these syllables immediately after receiving the LMAN lesions and not after the ts nerve injury; those two syllables were thus not included in the consideration of changes to songs after ts nerve injury. Overall, an average of $2.5 \pm 0.8$ syllables were added or lost in the songs of birds that received sham lesions prior to ts nerve injuries, while $0.375 \pm 0.183$ syllables were added or lost to songs of birds that had previously received LMAN lesions. This difference in total turnover of song syllables was statistically significant ($t = 2.584$, df $= 14$, $p < .05$). Comparisons across all treatment groups [Fig. 4(a)] indicate that an overall effect exists [$F(3, 24) = 6.808$, $p < .005$], and posthoc analysis shows that differences between groups are only significant for the sham/ts treatment (which differed from each of the other groups; Fisher's PLSD $p < .01$), while the LMAN/ts group is statistically indistinguishable from the two groups that did not receive ts nerve injuries.

The manner in which syllables were lost from the song differed for birds with LMAN lesions and sham lesions. After adjusting for changes in overall song tempo, it was possible to determine whether the time interval that the syllable had occupied with in the song had been deleted altogether [Fig. 5(a)] or replaced with a silent period corresponding to the syllable's preoperative length [Fig. 5(b)]. We considered all syllables that had not been lost from the ends or beginnings of the song (where the presence of a replacement silent interval is difficult to determine). Of the two such syllables lost from the LMAN/ts birds' songs, both were silenced, while 13 of the 14 syllables lost from the sham/ts birds' songs were deleted. Although the data are negative, it appears that birds that received LMAN lesions may silence but not delete syllables from their songs after ts nerve injury. However, the extent of the lesions was not closely related to whether syllables were lost from the songs of birds in the LMAN/ts group. The two birds that silenced syllables had nearly complete bilateral LMAN lesions; the maximum remaining volume of the LMAN nuclei was 6% of the control birds' average total LMAN volume.

All of the syllables added to songs after ts nerve injury were added to the ends of songs. Six of the seven added syllables were stacks, and one was a combination of a downsweep and a stack. All seven syllables were call-like in that they included only elements similar to those normally found in calls that are given by both males and females. No high notes or clicks remained in the songs of birds that received ts nerve injuries (Williams et al., 1992), and the distri-

**Table 1  Changes to Songs of Individual Birds after ts Nerve Injury**

| | Age (mo) at Time of Surgery | Syllables | | | | Changes as % of Syllables in Intact Song | Average Change in % of Songs Including Each Syllable |
|---|---|---|---|---|---|---|---|
| | | Intact Song | Final Song | Lost | Added | | |
| Birds with LMAN lesions | | | | | | | |
| W71 | 24 | 10 | 10 | 0 | 0 | 0 | 2 |
| O32 | 27 | 12 | 13 | 0 | 1 | 8.3 | 16 |
| O10 | 5 | 6 | 6 | 0 | 0 | 0 | 8 |
| Y46 | 14 | 12 | 11 | 1(1) | 0 | 8.3 | 20 |
| Y186 | 25 | 17 | 17 | 0 | 0 | 0 | 16 |
| W86 | 22 | 11 | 11 | 0 | 0 | 0 | 10 |
| DG22 | 37 | 10 | 9 | 1(1) | 0 | 10.0 | 14 |
| Bk66 | 33 | 12 | 10* | 0 | 0 | 0 | 21 |
| Mean $\pm$ S.E. | 23.4 $\pm$ 3.6 | 11.3 $\pm$ 1.1 | 10.8 $\pm$ 1.1 | 0.25 $\pm$ 0.2 | 0.125 $\pm$ 0.125 | 3.3 $\pm$ 1.6 | 13.4 $\pm$ 2.3 |
| Birds with sham lesions | | | | | | | |
| O7 | 5 | 13 | 15 | 0 | 2 | 15.4 | 13 |
| O33 | 26 | 11 | 6 | 6 | 1 | 63.6 | 43 |
| Pk28 | 26 | 13 | 13 | 0 | 0 | 0 | 32 |
| R65 | 51 | 11 | 7 | 4(1) | 0 | 36.4 | 39 |
| R71 | 47 | 10 | 9 | 2 | 1 | 30.0 | 37 |
| LB76 | 60 | 9 | 9 | 0 | 0 | 0 | 36 |
| DP29 | 4 | 15 | 17 | 0 | 2 | 13.3 | 29 |
| LB48 | 20 | 13 | 11 | 2 | 0 | 15.4 | 24 |
| Mean $\pm$ S.E. | 29.9 $\pm$ 7.4 | 11.9 $\pm$ 0.7 | 10.9 $\pm$ 1.5 | 1.75 $\pm$ 0.8 | 0.75 $\pm$ 0.31 | 21.8 $\pm$ 7.5 | 31.6 $\pm$ 3.4 |

Numbers in parentheses adjacent to the number of syllables lost denote the number of those lost syllables that were silenced. Means that were significantly different between the two groups ($p < .05$) are underlined.

* Bk66 lost two syllables after LMAN lesion and before ts nerve injury, and this syllable loss was not included in consideration of song changes consequent to ts nerve injury.

bution of syllable types within the songs of birds that received ts nerve injuries did not differ significantly in birds that received LMAN lesions and those that did not ($\chi^2 = 2.0$, df $= 2$, $p > .3$).

***Changes in the Proportion of Songs Including Syllables.*** Restricting the analysis of changes in the song to syllables lost or added overlooks instances in which syllables were present in the song but were sung much less (or more) often after surgery. For example, bird LB76 sang a sequence of three syllables at the beginning of his song in more than 90% of the songs sung preoperatively but in only 6% of the songs sung in the final four recording sessions (Fig. 6); since they were present, none of these syllables were scored as lost. This shift in the frequency with which syllables were included within songs at the end of the study [Fig. 4(b) and Table 1] was much larger for the sham/ts birds (31.6 ± 3.4%) than for the LMAN/ts birds (13.4 ± 2.3%) and was greater for both of the ts groups than for the birds that received no ts injury (7.5 ± 1.4% for

LMAN lesions and 7.0 ± 2.3% for sham). These differences among the four experimental groups were significant [$F(3, 24) = 20.451$, $p < .0001$]. Posthoc analysis showed that although the LMAN/ts birds had larger average changes than the two control groups, the only significant difference among the treatment groups was the sham/ts birds' larger change in the average frequency with which syllables were included in the song (Fisher's PLSD, $p < .001$). Hence, the shift in syllable representation within songs after ts nerve injury was significantly reduced by LMAN lesions.

***Changes in Motif Variants.*** The shift in syllables included within the song was reflected in a shift in the motif variants most often used by individual birds [Figs. 4(c) and 7]. Only 20.8 ± 10.3% of the sham/ts birds' most common motif variants in the final postoperative recording were included among their most common preoperative motif variants. In contrast, birds in the LMAN/ts group primarily retained 72.5
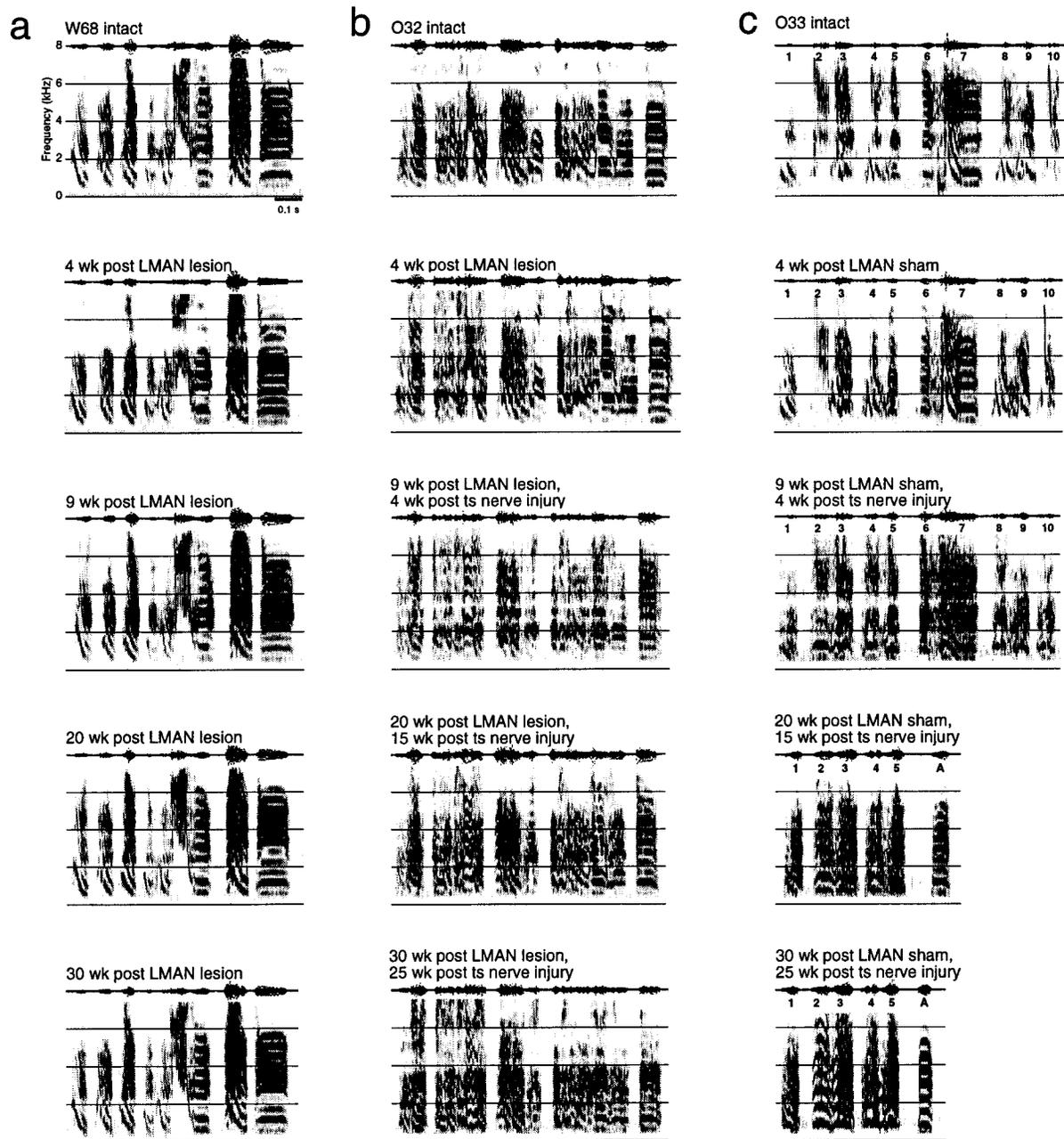
**Figure 3** Songs of representative birds during the course of the study. (a) W68 received bilateral lesions in LMAN and did not add or lose syllables or show changes to syllable phonology over the following 30 weeks. (b) O32 received bilateral lesions in LMAN followed by right ts nerve injury. Syllable phonology was affected by the ts nerve injury, but no syllables were lost or added to the song, and the structure of the song recorded 25 weeks later is readily seen to correspond to the preoperative song. (c) O33 had sham lesions followed by right ts nerve injury. Syllable phonology was affected by nerve injury, as can be seen in the recording made 4 weeks after nerve injury, and only the introductory note and the first four syllables were retained in the song in the recording made 15 weeks after nerve injury. A new syllable was added to the end of the song at this time, and the song remained stable during the next 10 weeks.

$\pm$ 11.1% of the motif variants that had been most common in their songs before surgery. Both ts nerve injury groups preserved fewer of their motif variants than birds in the sham (95.8 $\pm$ 4.2%) and LMAN (82.0 $\pm$ 5.9%) groups, and there was a significant difference among groups [$F(3, 24) = 12.976$, $p$
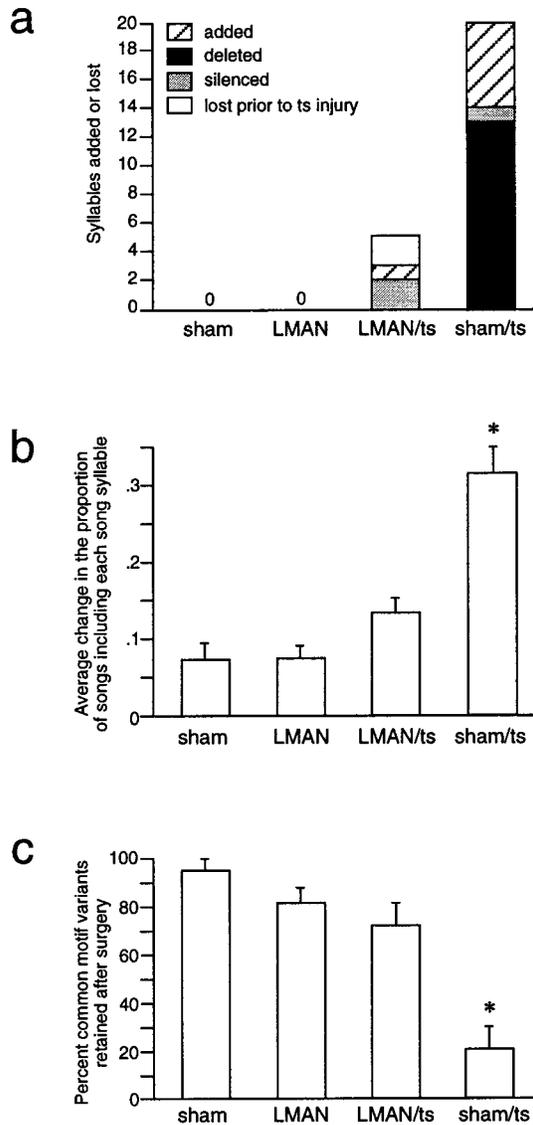
**Figure 4** Lesioning LMAN reduced the song changes that occur after ts nerve injury. (a) Birds from the sham/ts group added and lost more syllables than did birds in the LMAN/ts group. Lost syllables are divided into three categories: silenced, in which a syllable is removed from the song but is replaced by a silent interval similar in length to the original syllable; deleted, in which a syllable is removed from the song and no silent interval remains in its place, and lost prior to ts nerve injury, when a syllable was lost from the end of the song during the interval between LMAN lesion and ts nerve injury. (b) Birds from the sham/ts group changed the representation of syllables within their songs significantly more than did birds from the LMAN/ts group, which was statistically indistinguishable from the sham and LMAN groups. (c) Birds in the sham/ts group retained fewer of their original motif variants at the end of the study, while birds in the other groups did not differ statistically for this measure. Groups with bars denoted by an asterisk were significantly different from all other treatment groups, Fisher's PLSD, *p* < .001.

< .0001]. Posthoc tests showed that the birds in the sham/ts group preserved their motif variants significantly less often than did the birds in any of the other groups (Fisher's PLSD, *p* < .001) but none of the other differences between groups were significant. LMAN lesions diminished the shift in motif variants normally seen in birds with ts nerve injury.

***Lesion Size and Alterations to Song.*** The extent of the lesion, as judged by the volume of intact LMAN, was not significantly correlated to the number of syllables changed after ts nerve injury (*r* = .185, *n* = 8, *p* > .4) or to the shift in the average frequency with which syllables were included in the song (*r* = .001, *n* = 8, *p* > .99). However, it is possible that a more extensive examination of this specific question might show differential effects of lesion size and/or location within LMAN upon song.

***Measures of Directed Song.*** Undirected songs have more motif variants during a recording session (Sossinka and Böhner, 1980) and it is possible that the changes in song composition we observed over the course of the study were secondary to differences in the proportion of undirected songs sung by birds in the sham/ts group. Directed and undirected song also differ in the number of motifs that are sung in each strophe (more are sung in directed song) and in the number of introductory notes that precede each strophe (fewer are sung before directed song). A comparison of these measures during the final two recording sessions showed that although the sham/ts birds sang the fewest motifs/strophe (1.4) and the most introductory notes/strophe (2.8), there were no significant differences between treatment groups [*F*(3, 24) = 2.23, *p* > .1 for motifs per strophe; *F*(3, 24) = 0.789, *p* > .5 for introductory notes per strophe].

## DISCUSSION

### Effects of LMAN Lesions on Adult Zebra Finch Song

LMAN lesions in adult male zebra finches did not affect song syllables or the stereotypy of their order, confirming previous results (Bottjer et al., 1984; Scharff and Nottebohm, 1991; Nordeen and Nordeen, 1993). However, we found that LMAN lesions did in some cases appear to affect song tempo: songs of birds with bilateral lesions placed in LMAN were sung as much as 16% faster over the course of the study. Although only 3 of 14 birds shortened their songs by more than 10%, all birds with LMAN lesions sang their songs faster after surgery, and
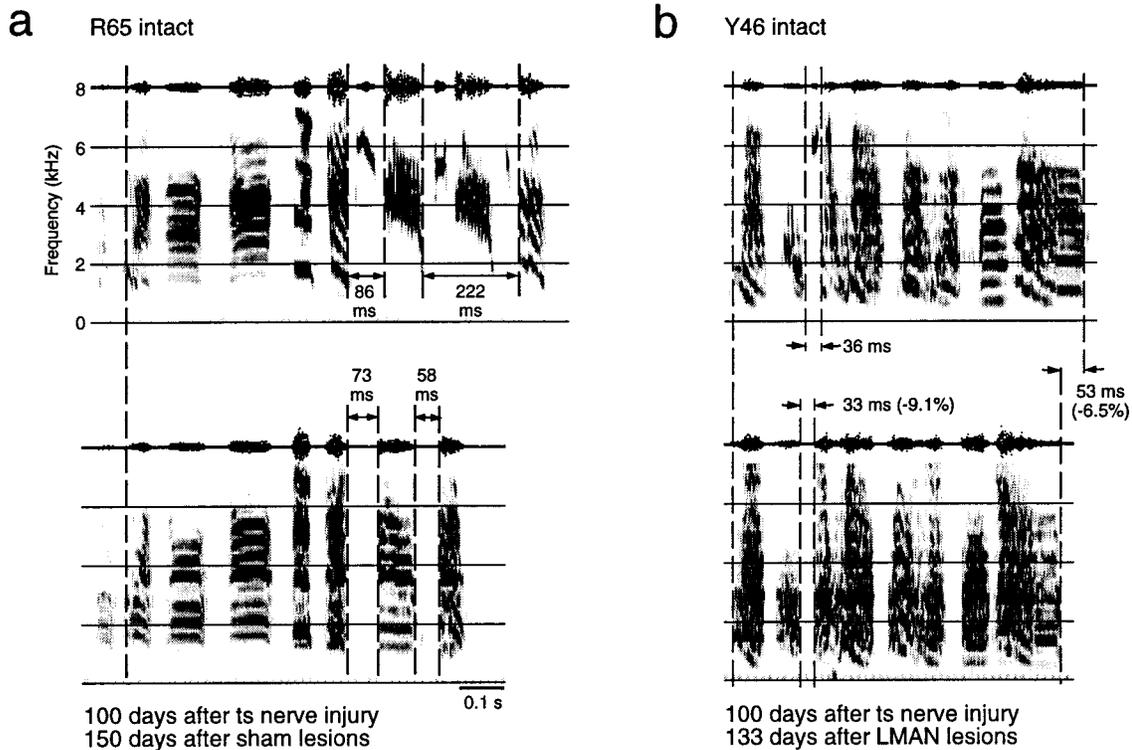
**Figure 5**  Birds with sham lesions and ts nerve injuries both silenced and deleted syllables, while LMAN/ts birds silenced syllables. (a) Example of a bird that silenced one syllable and deleted three syllables after sham lesions and ts nerve injury. The first five syllables of R65's song remained intact after sham lesions and ts nerve injury, although their acoustic structure was altered as a consequence of the ts nerve injury. The sixth syllable, a high note, was lost from the song, and the interval between syllables 5 snd 7 was reduced by 13 ms in the postoperative song, which was substantially less than the duration of the syllable, indicating that the syllable was silenced. The seventh syllable remained in the song, and the 8th, 9th, and 10th syllables (a short high note, a train of clicks, and a very short high note) were lost. The interval between syllables 7 and 11 was reduced by 164 ms, and syllable 11 was sung immediately after syllable 7, indicating that syllables 8, 9, and 10 were deleted. (b) Example of a silenced syllable in the song of Y46, a bird that received LMAN lesions and a ts nerve injury. At the end of the study, syllable 3, a short high note, no longer appeared in the song, and was replaced with a 33-ms silent interval betweeen syllables 2 and 4, a reduction of 9.1% in the time intervening between syllables 2 and 4 in the original song. This reduction was nearly identical to the overall reduction in song length of 6.5%, indicating that the syllable was silenced and not deleted from the song.

LMAN-lesioned birds increased their song tempo more than did sham-lesioned birds (although the trend did not reach significance). Previous lesion studies may not have measured song tempo systematically, or results similar to ours occurred but were not reported; alternatively, differences in the placement and size of lesions may yield different effects upon song tempo. Our lesions were placed slightly lateral to the center of the nucleus and did not impinge upon medial MAN. Hence, the change in tempo is unlikely to be mediated by direct lesion effects upon medial MAN, although we cannot rule out the possibility that connections between medial MAN and other nuclei in the song system were affected. Some of our lesions may have affected the dorsolateral margin of Area X, but our lesions of LMAN would most likely have affected Area X primarily by removing the input from LMAN (Nixdorf-Bergweiler et al., 1995; Vates and Nottebohm, 1995; Vates et al., 1997) and interrupting the lateral recursive loop, which carries the outputs of Area X (Area X projects to DLM which projects to LMAN) (Okuhata and N, 1987; Bottjer et al., 1989). One possible explanation for these results is that the changes in tempo are related to lesion-induced alterations in the directedness of song, as is suggested by the finding that singing undirected (but not directed) song increases ZENK expression in the Area X and LMAN of adult zebra finches (Jarvis, in press). How-
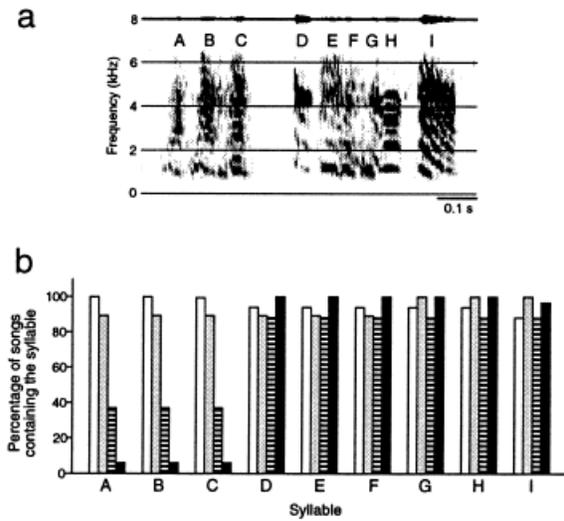
**Figure 6** The proportion of songs that contain a syllable or group of syllables that are not lost can change substantially. The song of LB76 initially contained nine syllables, here labeled A–I, all of which were included in at least 85% of the songs sung in the two preoperative recording sessions (white bars). Syllables D–I continued to be given within the same proportion of songs after sham lesions (stippled bars), 10 weeks after ts nerve injury (striped bars), and 20 weeks after ts nerve injury (black bars), but syllables A–C, which were given in 89% of the songs after sham lesions, occurred in only 6% of the songs at the end of the study. Although these syllables were not silenced or deleted, it is clear that their representation within the song was reduced. In calculating the average change in proportion of songs included in a song, each of these syllables would receive a score of 0.94 (the absolute value of the difference between 1.00, the proportion of preoperative songs containing the syllable and 0.06, the proportion of songs containing the syllable during the final recording). For this bird, the average change in the inclusion of syllables was 0.36 (introductory notes are not included in this measure).

ever, two song measures that distinguish between directed and undirected song (introductory notes per strophe and motifs per strophe) did not change in birds that increased song tempo.

If the change in song tempo is indeed a consequence of lesioning LMAN, studies of the topographical organization of LMAN suggest a possible anatomical correlate. The core of LMAN projects primarily to the ventral two thirds of RA (Vates and Nottebohm, 1995), and a region surrounding LMAN, or shell, projects to an area immediately adjacent to RA (Johnson et al., 1995). Perhaps lesioning the LMAN shell could affect aspects of song timing by means of the respiratory branch (Vicario, 1993) of the song control system. Although we do not know whether LMAN has a role in establishing song tempo during song development, our results indicate that

LMAN may play a role in song tempo after song crystallization.

## LMAN Lesions Decrease Song Changes Induced by ts Nerve Injury

Birds with sham lesions showed substantial quantitative and qualitative differences in their songs after ts nerve injury, confirming previous work (Williams and McKibben, 1992). The eight birds in this group silenced one syllable, lost 13 syllables, and added seven syllables to their songs, and had significantly greater shifts in syllable representation within their songs and in common motif variants than did birds in the control groups. In contrast, bilateral lesions placed in LMAN largely prevented the song changes that normally follow ts nerve injury. The songs of the eight lesioned birds lost only two syllables and added but one syllable. Birds in the LMAN/ts group also maintained the preoperative representation of syllables within their songs and used the same motif variants in their songs before and after surgery. Syllables lost from the songs of LMAN-lesioned birds after ts nerve injury were more likely to be silenced than deleted. The slight increases in changes to the songs of birds in the LMAN/ts group as compared to the two control groups (LMAN- and sham-lesioned birds) in the average proportion of songs containing each syllable and in the motif variants most often sung, although not statistically significant, suggest that incomplete lesions of LMAN may have allowed some changes to song. Although there was no relationship between the volume of LMAN that was spared and the degree of change to the song, it is possible that if different portions of LMAN subserve different functions, measures of LMAN volume do not reflect the severity of the lesion from a functional standpoint. However, the trend is clear: With the possible exception of silenced syllables, the birds with LMAN lesions maintained their songs after ts nerve injuries, while birds with sham lesions made substantial alterations to their songs.

Since LMAN is not required for maintaining adult song in zebra finches but is necessary for normal song development, it appears that to some extent the role of LMAN after ts nerve injury reverts to one similar to that played by the nucleus during song development. However, the song changes that occur after ts nerve injury cannot be described as song learning, but are a more limited form of plasticity that reconfigures an existing vocalization rather than developing a novel song based on imitation of a model. Although we cannot rule out the possibility that right ts nerve injury affects an adult male's capacity to generate novel syllables, it seems unlikely that the limitations on
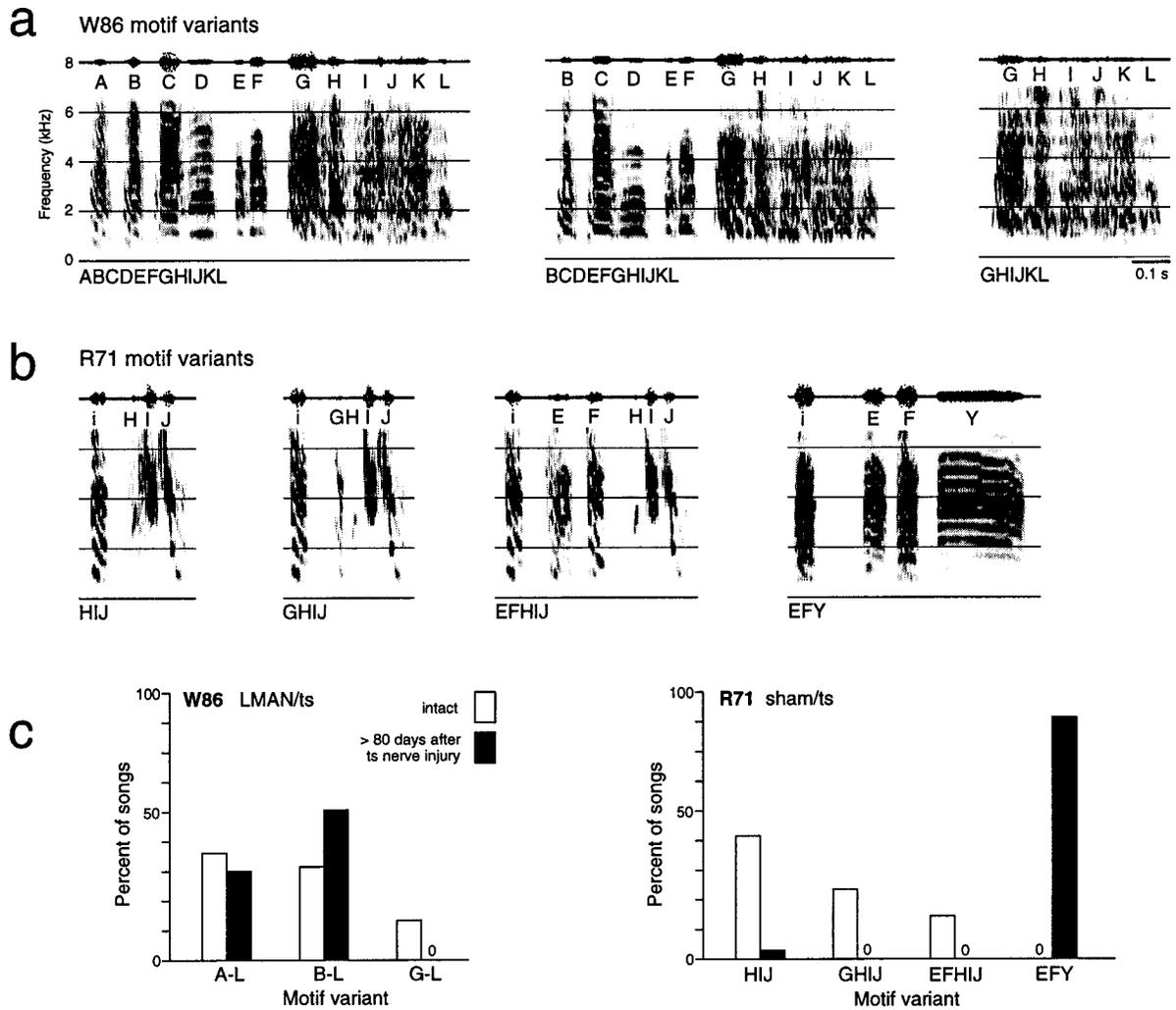
**Figure 7** Changes in motif variants. (a) The most common motif variants used by W86 before and after LMAN lesions and ts nerve injury. The full song included 12 syllables (A–L); a second motif variant omitted the first syllable (B–L), and the third most common motif variant consisted of the final six syllables (G–L). No introductory notes are shown. (b) Four motif variants sung by R71, a bird that received sham lesions followed by ts nerve injury. The most common preoperative motif variant was a short sequence of three notes (HIJ). Another common preoperative motif variant was the same sequence preceded by another syllable (GHIJ), and the third most common preoperative motif variant was the sequence EFHIJ. There were other, less common motif variants including ABCDEFHIJ (syllable G only occurred in motif variant GHIJ). At the end of the study, the most common motif variant included a new syllable (Y), appended to EF. The song type HIJ was retained, as were some rare motif variants (such as BCDEFY, not shown here), which allowed us to unequivocally designate syllable Y as a novel syllable and the sequence EFY as a novel motif variant. (c) Comparison of the motif variants used in preoperative song and the songs sung at the end of the study. Taken together, the three motif variants shown in (a) accounted for 90% of the songs sung by W86 during the two initial recording sessions. During the two final recording sessions (>80 days after ts nerve injury), variant G–L was not sung, but variants A–L and B–L accounted for 89% of all of the songs; thus, all of the most common variants in these final postoperative session motif variants were also among the most common preoperative variants. In contrast, the three most common motif variants given by R71 during the initial recordings accounted for 88% of all songs, but those three motif variants made up only 3% of the songs given in the final two sessions after surgery. The novel motif variant, EFY, accounted for 90% of all songs in those recording sessions.

adult plasticity are entirely due to the ts nerve injury: Young birds develop songs that fall into the normal range after right ts denervation (H. Williams, unpublished data). Although other explanations are possible, we favor the hypothesis that the neural changes in the song system that occur during development, perhaps specifically those that take place in LMAN, reduce the possible outcomes of song plasticity.

Anatomical and pharmacological studies of LMAN during song development would tend to support this interpretation: LMAN decreases in volume (Bottjer and Sengelaub, 1989; but see Nordeen et al., 1992) as does the branching of distal dendrites and the density of dendritic spines upon LMAN neurons (Nixdorf-Bergweiler et al., 1995); the concentration of NMDA receptors within LMAN also decreases (Aamodt et al., 1992); blockade of NMDA receptors in LMAN prior to crystallization disrupts song learning (Basham et al., 1996); and the specificity of auditory responses within LMAN increases during song development (Doupe, 1997). The trajectory of some of these parallels to song development can be altered by prolonging the sensitive period for song learning: Depriving zebra finches of a song model prevents the loss of dendritic spines within LMAN (Wallhausser-Franke et al., 1995). If at least some of the anatomical and physiological changes that occur in LMAN during zebra finch song development are irreversible, they may underlie the reduced possibility for plasticity in adults that we observed here and that also was seen in previous studies (Williams and McKibben, 1992).

We do not yet know precisely what role LMAN plays in reconfiguring crystallized adult zebra finch song. Song-selective neurons in LMAN gain selectivity and specificity for the bird's own song during development (Doupe, 1997), LMAN neurons are active during song production in adults (Hessler and Doupe, 1997) and LMAN lesions impair zebra finches' ability to discriminate among conspecific songs (Scharff et al., 1998). It is possible that the change in syllable phonology consequent to ts nerve injury yields a mismatch between actual and expected vocal output that is registered as a sensorimotor mismatch within LMAN; if so, lesions within LMAN might reduce the chance that such a mismatch would trigger compensatory changes to the song. The recent observation (Jarvis et al., 1998) that undirected and directed song in adult male finches are differentiated not only by the presence or absence of an object for the song but also by the expression of an immediate early gene in Area X and LMAN (which occurs only after undirected song) is intriguing. Perhaps undirected song and the specific neuronal events that ensue within LMAN are necessary for any changes to

song both during development and in altering crystallized song. Although the specific mechanisms are not clear, LMAN can play a role in both adult song plasticity and normal song development.

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