

Sensitive Period for Sensorimotor Integration during Vocal Motor Learning

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ABSTRACT: Sensory experience during sensitive periods in development may direct the organization of neural substrates, thereby permanently influencing subsequent adult behavior. We report a sensitive period during the imitative motor learning phase of sensorimotor integration in birdsong development. By temporarily and reversibly blocking efference to the vocal muscles, we disrupted vocal motor practice during selected stages of song development. Motor disruption during prolonged periods early in development, which allows recovery of vocal control prior to the onset of adult song, has no effect on adult song production. However, song disruption late in development, during the emergence of adult song, results in permanent motor defects in adult

song production. These results reveal a decreased ability to compensate for interference with motor function when disturbances occur during the terminal stage of vocal motor development. Temporary disruption of syringeal motor control in adults does not produce permanent changes in song production. Permanent vocal aberrations in juveniles are evident exclusively in learned song elements rather than nonlearned calls, suggesting that the sensitive period is associated with motor learning. © 2000 John Wiley & Sons, Inc. *J Neurobiol* 42: 172–189, 2000

Keywords: sensitive period; sensorimotor integration; motor learning; song development; zebra finch

Song learning by oscine songbirds is functionally divided into two phases: memorization of adult conspecific model songs (sensory phase), and modification of vocal output to produce a copy of the model songs (sensorimotor phase) (Konishi, 1965). The existence of a sensitive period within the sensory phase has been demonstrated by exposing juveniles to model songs during specific periods throughout song development (Eales, 1985, 1987; Clayton, 1987; Bohner, 1990). By manipulating the timing as well as the conditions under which a model is available, sensitive periods for the sensory

phase have been shown to vary across species and to be influenced by social (Baptista et al., 1984, 1986; Kroodsma et al., 1984; Eales, 1987; Slater et al., 1988; Bohner, 1990; Jones et al., 1996), environmental (Kroodsma et al., 1984), and physiological conditions (Nottebohm, 1969; Arnold, 1975; Prove, 1983, 1985; Morrison et al., 1993).

The sensorimotor phase is broadly divided into the stages of subsong and plastic song production, and is likely to function at least in part as a period of motor practice. Subsong vocalizations, beginning in zebra finches by about day 30, are characterized by low-amplitude, highly variable sounds bearing little resemblance to the adult song that will eventually be produced. Early in plastic song production, by about day 50, vocalizations become louder, increasingly stereotyped, and recognizable as components of adult song. Late in the plastic song stage, there is selective attrition of song elements which are dropped from the repertoire. Syllable structure, sequence, and song temporal pattern become stereotyped. The emergence of

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stereotyped adult song during song crystallization characterizes the establishment of the adult motor pattern, which in zebra finches occurs at around 90 days of age.

During song development, auditory feedback from the juvenile's vocalizations is thought to be compared with the stored memory of the model's song. The output of these comparisons presumably modifies subsequent motor commands in such a way as to successively increase the similarity between the bird's own song and that of the model. Sensorimotor integration also requires that an association between auditory feedback and the initiating motor pattern is maintained long enough to allow the shaping of motor commands. The role of proprioceptive feedback from the vocal apparatus during song learning has yet to be determined (Bottjer et al., 1984, 1986).

Whereas varying juvenile exposure to model songs has resulted in the identification of a sensitive period during the sensory phase, the lack of a technique to reversibly manipulate juvenile vocalizations, syringeal feedback, or auditory feedback has prevented similar advances in the study of sensitive periods within the sensorimotor phase. Interestingly, preventing the secretion of gonadal hormones prior to the onset of subsong (Nottebohm, 1969) as well as rearing in an abnormal social context (which may similarly prevent normal hormone production) (Morrison et al., 1993) will delay both the sensory and sensorimotor phases of song learning past the usual developmental period. However, it remains to be determined whether, given a normal social and hormonal environment, a developmental sensitive period exists during the phase of sensorimotor integration.

Here we present the results of reversibly disrupting vocal motor control during various stages of song motor development, allowing normal vocal production both prior to and following the periods of manipulation. Vocalizations were disrupted for periods of weeks in juvenile male zebra finches by botulinum toxin (BoTX) injected into the muscles of the syrinx, the avian vocal organ. BoTX produces skeletal muscle paralysis by preventing the release of acetylcholine at the neuromuscular junction (Simpson, 1981). Recovery of muscle function occurs with the formation of newly active motor end plates (Duchen, 1971). During the period of paralysis, auditory and presumably syringeal feedback are mismatched with the initiating motor commands.

We found that interference with sensorimotor integration during early periods of song practice has no detectable effect on normal song development if motor function recovers prior to the emergence of adult song. However, syringeal paralysis occurring late in development, during the time at which adult song

appears, results in irreversible impairments in song production. We conclude that the differences in ability to recover from sensorimotor disruption early and late in development indicate a sensitive period late in the sensorimotor phase of song development.

MATERIALS AND METHODS

Housing

Zebra finches were hatched in our laboratory breeding colony and maintained on a 12:12-light/dark cycle throughout the year. Zebra finch song is produced only by males. Juveniles tend to copy song elements from their father, the song tutor, and produce a single stereotyped song. Juvenile males were reared in one of two conditions: in a family cage ($n = 42$ juveniles) or in their natal aviary ($n = 32$). Cage-reared juveniles were removed from the colony at or before the time of fledging [range, 14–36 days old; mean, 25; standard error (SE), 0.91] and each clutch was placed in a cage ($44 \times 44 \times 56$ cm) with an adult male tutor and his mate. Female siblings were removed from these cages between the ages of 35 and 40 days. All cages were housed in a single room with auditory and visual contact among families for the duration of the experiment. Ten additional experimental clutches were reared by their respective parents in a single aviary.

Clutches contained two to six male siblings. Within each cage- or aviary-reared clutch, one or two males were designated as control birds ($n = 28$) and, when possible, experimental males were assigned to different treatment groups. Thus, differences in results among treatments could not be ascribed to differences in tutoring or rearing conditions. Adult control males were given BoTX treatments to evaluate recovery of syringeal function following reversal of muscle paralysis and to control for surgical effects of the injections. The adult controls (>1 year old, $n = 18$) were taken from the colony and housed throughout the experiment in five groups of three to five individuals under the same conditions as the family cage groups.

Treatment Groups

Juveniles were assigned to one of six experimental groups determined by the stage(s) of song development at which they received BoTX injections (Fig. 1 and Table 1). Three groups of juveniles received a single dose of BoTX injected into syringeal muscles at one of the following developmental stages: (a) prior to or early in subsong (S), (b) during plastic song (P), or (c) prior to song crystallization (C). Three other

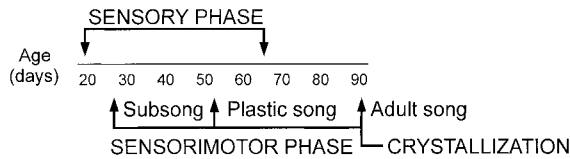


Figure 1 Stages in song learning. Memorization of a tutor's song during the sensory phase occurs between approximately days 20–65. The sensorimotor phase includes the juvenile subsong and plastic song vocal stages. Plastic song increasingly resembles the song of the juvenile's tutor, becomes adultlike by about day 80, and crystallizes during approximately days 90–100.

treatment groups received BoTX at two times during song development, one dose at each of the following stages: (d) early in subsong and during plastic song (S + P), (e) during plastic song and prior to the usual age of song crystallization (P + C), and (f) early in subsong and prior to the age of song crystallization (S + C). Song stages were determined by age, not by the juveniles' vocalizations.

Adult Controls

Adult controls received the same dosage of BoTX separated by the same time intervals as those given to the juveniles: either a single injection ($n = 7$) or two injections 28–29 days apart ($n = 6$) or 50–55 days apart ($n = 5$). Six adults receiving two injections recovered normal vocalizations prior to the second injection. An analysis of the songs of these birds before and after the first treatment is included in the

results of the single treatment adults (total $n = 13$) (Table 1).

Juvenile Controls

Songs of juvenile control siblings raised with experimental juveniles were used as the standard for measurements of song learning and song production within the laboratory or aviary environment.

Botulinum Toxin Injections

Zebra finches were anesthetized with intramuscular injections of chloropent (3.5 $\mu\text{L/g}$; Fort Dodge Laboratories, Fort Dodge, IA) and the syringe was exposed through an incision in the interclavicular air sac. Lyophilized BoTX type A supplied as 100 MU Botox[®] (Allergan) was reconstituted in 0.33 mL sterile buffered 0.9% saline, and a total of 1.0 μL of the suspension was injected bilaterally into ventral and ventrolateral syringeal muscles (mm. syringealis ventralis, s. tracheobronchialis, and s. tracheolateralis) using a pressurized microinjection system (Picospritzer[®] II; General Valve Co.) coupled to a glass micropipette. Multiple injections were made bilaterally (four to six on each side), distributed evenly throughout the ventral syringeal muscle mass. An epoxy bead placed 1.5 mm from the tip of each micropipette ensured that injection depths were consistent across birds.

Table 1 Age and Song Stages at BoTX Injections

Treatment Group	Song Stages	Age When Injected [range (days)]	Interval between Injections [range, (days)]	n_{exp}^*	n_{ctl}
Juvenile single injection	Subsong (S)	21–32	Single injection	7	5
	Plastic (P)	48–58	Single injection	8	6
	Crystallization (C)	71–87	Single injection	9	2
Juvenile double injection	Subsong + plastic (S + P)	25–39 and 53–67	28–29	8	4
	Subsong + crystallization (S + C)[†]	25–34 and 79–87	53–55	6	6
	Plastic + crystallization (P + C)	50–65 and 78–93	28–29	8	5
Adult single injection	Postcrystallization	>90	Single injection	13	
Adult double injection [‡]	Postcrystallization	>90	28–29	6	
	Postcrystallization	>90	50–55	5	

* Sibling controls were reared with each juvenile treatment group (n_{exp} = number of experimental birds; n_{ctl} = number of nontreated sibling controls).

[†] Bold type indicates treatments which resulted in the development of abnormal adult songs.

[‡] Adults received the same dosage of BoTX at the same injection intervals as juveniles: 28–29 days apart, or 50–55 days apart.

Recordings

Recordings were made with a Sennheiser microphone (MKH416 TU-3) and Marantz cassette recorder (PMD 221), interfaced with a customized digital delay (RDS 4000; DigiTech International, Amherst, NH) and sound-activated switch (Akustomat F 413 Report Monitor; Uher). All songs recorded were directed song (Sossinka et al., 1980). Birds were recorded daily for 1 week prior to each surgery and throughout the period of paralysis. Thereafter, they were recorded weekly until 80 days of age, when they were again recorded daily until song crystallization was confirmed by the production of several days of invariant songs. Control juvenile siblings were recorded at the same ages as experimental birds. A minimum of 30 songs was recorded for each bird in each taping session. Following crystallization, songs were recorded once a month for 9 months. Spectrographs were generated with a Kay Sonagraph 6061B (frequency range 1–8 kHz, bandwidth 300 Hz, flat response).

Song Analysis

Zebra finch song consists of a single introductory syllable which may be repeated a variable number of times, followed by a stereotyped sequence or motif, usually composed of different syllables. Occasionally, the same syllable may occur more than once in the motif. Motifs are repeated, often separated by one or more introductory syllables, constituting a song bout.

A syllable is defined as a continuous trace on a spectrograph, delineated by silent intervals, and often consisting of more than one note. A note is a unit of a single type of acoustic structure (e.g., Fig. 4). Adjacent notes forming a syllable are identified by a disjunction in acoustic structure. The acoustic structure of notes is classified into one of the five following categories (modified from Price, 1979; Williams et al., 1992); (a) harmonic stack: composed of multiple harmonics with little or no frequency modulation, (b) frequency modulated; consists of rapidly changing frequencies, (c) click: a short duration broad band syllable, appearing as a single vertical mark on a spectrograph, (d) striated: similar to a click in structure but longer in duration, (e) other: any note which does not meet the criteria for one of the above categories.

Criteria for Recovery from BoTX

Following BoTX injections, song elements were produced as bursts of broadband noise with a characteristic fricative quality. Recovery from syrinxal paral-

ysis was evident in the loss of fricative structure and the reemergence of differentiated, acoustically complex, and frequency modulated components.

Song Measurements Posttreatment: Comparisons between Experimental and Control Adult Songs

The number of notes and syllables per song, acoustic category of notes, and note copying were evaluated by visual inspection of spectrographs by two independent observers. Both were blind to the bird's identity and treatment group. One observer was familiar with zebra finch spectrographs and the experimental design; the other was naive to spectrographs and had no a priori expectations of song copying or adult song stereotype. Both sets of data yielded the same statistical similarities and differences among groups.

All song measurements were made on crystallized adult songs, after the effects of paralysis were no longer detected in the acoustic structure of the spectrograms. Five or more songs per individual were used for quantification. Only complete songs were measured (as in Eales, 1985; Clayton, 1987; Morrison et al., 1993). Introductory syllables and syllables repeated in the motif were counted only once. The number of notes present in a song was counted, rather than the number of syllables, as this provides a more accurate measure of song complexity. Copying was calculated as the percentage of notes in the son's song that were derived from the tutor. Because individual notes can be copied from the tutor and reproduced in different syllables, the number of notes copied, rather than syllables, provides a more sensitive index of copying ability. The assessment of note matching between juveniles and tutors follows that of Morrison and Nottebohm (1993).

Song Measurements Pre- and Posttreatment: Comparisons within Individuals during Song Development

Pre- and posttreatment comparisons of spectrographs within individuals were made by the two observers noted above. In addition to the song measurements described, we recorded the numbers of song syllables added, deleted, or changed in acoustic structure following recovery from paralysis. Syllable additions, deletions, and changes were scored only in the three groups with a crystallization-period treatment (C, S + C, and P + C), following the criteria used by Williams (1990) and Williams and McKibben (1992). A note addition was reported when a pretreatment silent interval was occupied by a posttreatment song element. When temporal changes prevented the align-

ment of pre- and posttreatment songs, an addition was reported if a posttreatment note occurred without the same note present anywhere in the pretreatment song. Syllable deletions were scored when a pretreatment syllable was replaced by a posttreatment silent interval. Syllable changes were reported when pre- and posttreatment songs could be aligned temporally and syllables with different acoustic structure were located in the same position before and after treatment. Only major changes in acoustic structure were noted. In no case was a syllable change recorded as a syllable deletion followed by a syllable addition. Changes in temporal pattern were also reported. Pre- and posttreatment changes in the note-type composition of songs are described. The same measurements were taken from songs of sibling controls at the same ages as the experimental birds.

Call Analysis

A nonlearned, short-duration, low-amplitude call, often similar in structure to introductory notes, is frequently given while a zebra finch is within close range of flock members (Zann, 1996). Spectrographs of these call notes ($n = 6-10$ examples/bird) for each individual in groups C, S + C, and P + C were examined prior to crystallization-period treatments, during postinjection paralysis, and following syringeal recovery, by the two independent observers described above.

Histology

Bilateral injections of horseradish peroxidase conjugated to cholera toxin (BHRP, 0.5 μ L 0.2% solution; List Biological) were made into the ventral and ventrolateral syringeal muscles of two birds with the most severe adult song aberrations (one from group S + C and one from group P + C) and in three adult nontreated birds. In the treatment birds, the tracer was injected approximately 2 months after the reversal of syringeal paralysis. After a 48-h survival period, birds were anesthetized with chloropent and perfused intracardially with saline followed by 1% paraformaldehyde/1.25% glutaraldehyde. The brains were frozen-sectioned in a transverse plane at 30 μ m. The tissue was reacted using tetramethyl benzidine (TMB; Sigma) and counterstained with Neutral Red. Nucleus XII volume was estimated based on section thickness and cross-sectional areas which were measured using a projection microscope and digitizing tablet. The number of BHRP-filled neurons in nXII was counted under brightfield illumination ($\times 20-40$ magnification) in a sample of 7 sections/side per bird. Nonlabeled cells were counted in the same sections, and the

percentage of labeled cells was calculated. Mean neuronal density was calculated by counting neuron number in sections throughout nXII and measuring the corresponding section area. Total nXII neuronal number was estimated by multiplying nuclear volume by average neuron density. Measurements of left and right sides were combined in determining means. Syringeal muscle size of experimental and nontreated birds was visually compared. The muscle mass of these syrinxes was also visually compared with that of five adults that had undergone permanent syringeal denervation by bilateral NXIIts section early in plastic song, at approximately day 40.

Statistical Analyses

The following descriptive measurements are presented as mean \pm S.E.: (a) number of notes per song, (b) percentage of the son's notes copied from the tutor, (c) syllable duration, and (d) the note/syllable ratio. Within each of these categories, comparisons were made (a) between adult songs of experimental birds and their sibling controls, and (b) within individual experimental birds before and after treatment. Song changes within experimental birds were then compared with song changes within sibling controls at the same ages. Differences in means between groups were assessed with the unpaired two-way t test. Differences in means within individuals were assessed with the paired two-way t test. The percentage of notes copied from the tutor was not recorded before the BoTX treatment in late plastic song because small changes in note structure prior to crystallization give a deceptively low pretreatment percentage of notes copied in both experimental and control birds.

In one of the treatment groups (P + C), changes in syllable duration following recovery was accompanied by an increase in variance, determined by the Levene's test for homogeneity of variances, and therefore the t test could not be used. In this case, we used the Wilcoxon matched-pairs signed-ranks test to compare duration means. The mean (\pm S.E.) nucleus volume, neuronal density, and neuron number were compared between experimental and control groups with the unpaired two-way t test.

The types of changes in songs pre- and postcrystallization (notes added, deleted, and changed in acoustic structure) are reported as the mean (\pm S.E.) percentage of song changed. Although these measurements were taken near the end of song development, small changes in songs continue to occur during this time in nontreated birds until song crystallization is completed. Therefore, measures of note additions, deletions, and changes in the songs of experimental birds are meaningless unless they are compared with

song changes in sibling controls during the same time period. The comparison between experimental and control means was made with the unpaired two-way t test. Differences in note composition pre- and post-treatment was assessed with the χ^2 test.

RESULTS

Botulinum toxin injected bilaterally into syringeal muscles produced characteristic effects on the vocalizations of both juveniles and adults. During syringeal paralysis, the acoustic structure of songs and calls was reduced to broadband noise. Vocal impairment was apparent within 24 h of the injection and, at the dosages used in this study, persisted for 12–24 days. The vocal effects of paralysis disappeared with the recovery of syringeal function (Simpson, 1981). The gradual functional recovery resulted in abnormal and inconsistent auditory, and probably proprioceptive, feedback that changed during reversal of paralysis. This altered feedback presumably interferes with the establishment of sensorimotor mapping based on a predictable association between motor commands to the syrinx and the resulting sensory feedback.

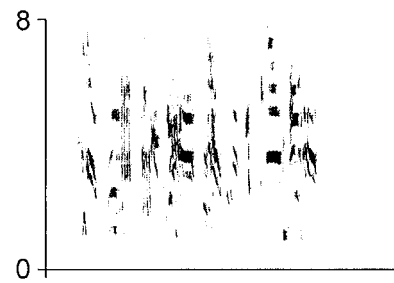
Adult Controls

Following recovery from syringeal paralysis, song returned to normal as determined by spectrographic analysis. After recovery from one or two periods of paralysis, 215 of a total of 221 (97%) of the notes produced by 24 adult birds were restored completely and were indistinguishable from pretreatment notes (Fig. 2).

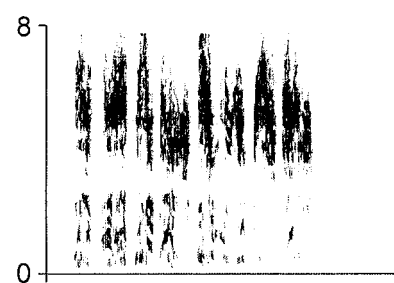
Early Motor Disruption: Paralysis during Subsong and Plastic Song

Comparison between Adult Songs of Experimental and Control Birds. The adult songs of birds given a BoTX treatment as juveniles during either subsong (S) or plastic song (P) did not differ from those of their respective sibling controls (Fig. 3). Furthermore, two successive BoTX treatments, during both subsong and plastic song (S + P), likewise had no detectable effect on the subsequent adult song. Following the return of syringeal function, these birds produced plastic songs which matured and crystallized into species-typical adult songs, similar to sibling controls in all parameters measured. There were no irregular features of song organization, temporal pattern, or acoustic structure distinguishing the songs of birds in this group from those of their sibling controls (Figs. 4 and 5).

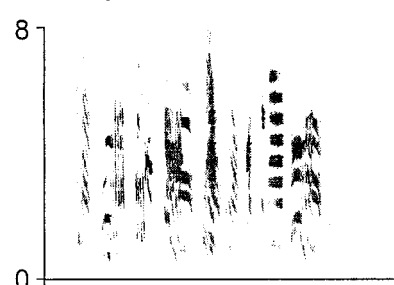
a) Pre-treatment adult song



b) 7 days post treatment 1



c) 30 days post treatment 1



d) 30 days post treatment 2

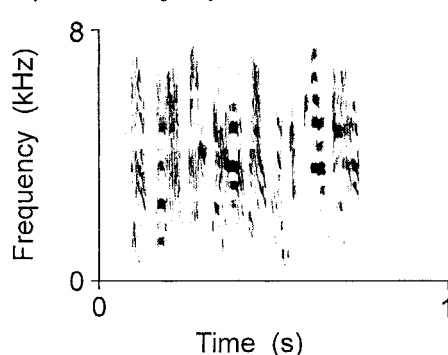


Figure 2 Reversible paralysis in adults. Bilateral injections of BoTX into the muscles of the adult syrinx causes song (a) to become noisy (b). The temporal pattern is not affected. The adult reproduces the preoperative song following recovery from a single BoTX treatment (c), and again following recovery from a second BoTX treatment 30 days after the first (d).

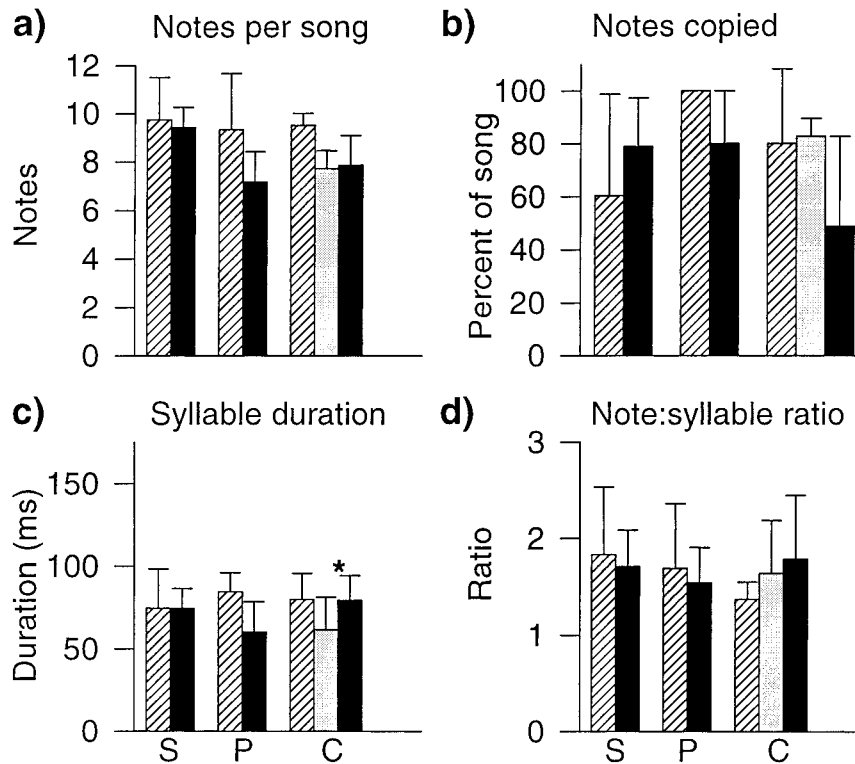


Figure 3 Song measurements of birds with a single BoTX treatment. Adult song characteristics (mean + S.E.) of experimental birds (black bars) given a BoTX injection either during subsong (S) plastic song (P) or prior to crystallization (C) are shown with nontreated sibling controls (hatched bars). Measurements of songs produced pretreatment (gray bars) are shown for the experimental birds in group C. Differences between songs of experimental birds and control siblings are not statistically significant (unpaired t test, $p > .05$). In the C group, mean posttreatment syllable duration is significantly greater than pretreatment duration (paired t test, $p < .05$).

Comparison within Individuals Pre- and Posttreatment. Within individuals, pre- and posttreatment comparisons could not be made in juveniles with BoTX treatments during subsong or plastic song since pretreatment songs were too variable and poorly defined to measure song characteristics. Although as a group, adult songs of treatment birds are indistinguishable from those of sibling controls, the possibility of within-individual treatment effects cannot be dismissed.

Late Motor Disruption: Paralysis during Crystallization

Comparison between Adult Songs of Experimental and Control Birds. Juveniles which received a single BoTX treatment late in song development, causing paralysis prior to and extending through the usual period of song crystallization (C), produced normal adult songs that had species-typical song organiza-

tion, temporal pattern, and acoustic structure, like those of sibling controls.

Comparison within Individuals Pre- and Posttreatment. Songs produced immediately prior to crystallization (at about day 80) in nontreated zebra finches were nearly stable in structure and changed little from this time through crystallization. Therefore, unlike the groups with early BoTX injections, within-bird comparisons of songs could be made before and after crystallization paralysis (C). Following recovery from the C period of paralysis, songs exhibited marked and permanent changes from the pretreatment songs. Posttreatment songs included new notes, whereas pretreatment notes were deleted or altered (Tables 2 and 3, and Fig. 6). The mean syllable duration was also changed (Fig. 3). Such changes did not occur in the songs of control birds recorded over the same time period (Table 2), indicating that there was an effect of the BoTX treatment during song crystallization, al-

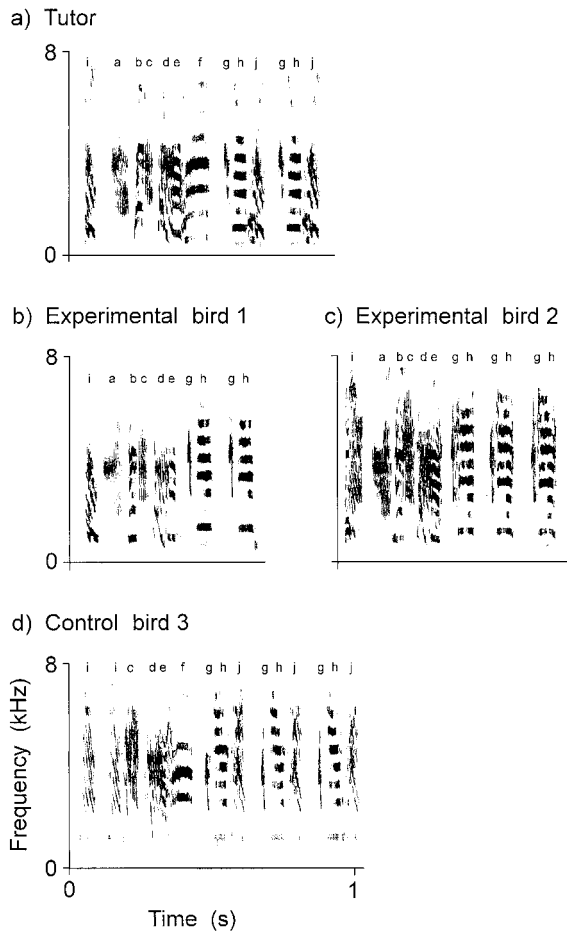


Figure 4 Spectrographs of the song of the tutor and the adult songs of two experimental and one control offspring (all are siblings) in group S + P. Experimental birds received a BoTX injection during subsong and plastic song (bird 1 at ages 28 and 56 days, bird 2 at 25 and 53 days). Songs were recorded following recovery from syringeal paralysis and after song crystallization, at day 96. The adult songs of experimental birds do not reveal effects of the juvenile BoTX treatment. Introductory syllables are denoted by “i.” Shared notes in the song motif are indicated by the same letters other than “i.” The notes “d” and “e” are examples of two notes forming a single syllable.

though the treatment did not result in an aberrant acoustic structure or temporal pattern.

Paralysis during Subsong and Crystallization

Comparison between Adult Songs of Experimental and Control Birds. Unlike the normal adult songs produced by birds with syringeal paralysis during either subsong or crystallization, a BoTX treatment during subsong followed by a second treatment during crystallization (S + C) resulted in distinctly abnormal

adult songs comprised primarily of harmonic stack notes (Fig. 7). These songs also contained proportionately fewer notes copied from the tutor than did the songs of control siblings (Fig. 5).

Comparison within Individuals Pre- and Posttreatment. Prior to the C treatment, S + C songs produced late in plastic song were no different from those of sibling controls recorded at the same age. This was consistent with the normal song development in birds with a single S treatment. Following recovery from the C period of paralysis, however, more than one third of the notes were changed in acoustic structure (Fig. 6 and Tables 2 and 3), preoperative notes were deleted, and new notes were added. These changes resulted in a net decrease in the mean number of notes per song [Fig. 5(a) and Table 2] a lower proportion of notes copied from the tutor [Fig. 5(b)], and an increase in mean syllable duration [Fig. 5(c)]. Of the 12 notes deleted following recovery from treatment, six occurred with the loss of an intersyllable interval, causing a change in song temporal pattern. None of the added syllables altered the song’s temporal pattern. Songs of sibling controls changed little over the same age interval (Table 2).

Paralysis during Plastic Song and Crystallization

Comparison between Adult Songs of Experimental and Control Birds. The most severe abnormalities in adult song structure occurred in birds that received a BoTX treatment during plastic song followed by a second treatment prior to crystallization (P + C). Compared with sibling controls, these experimental birds produced fewer notes per song, copied fewer notes from their tutor, had a greater variance of syllable duration, and produced syllables with a higher note/syllable ratio (Fig. 5). Unlike the S + C group, the majority of notes produced by P + C birds were strikingly atypical. In particular, long-duration striated notes (74–223 ms) (Fig. 8) and long-duration multinote syllables (composed of three to five different notes) occurred significantly more often in songs of P + C birds than in those of control siblings ($\chi^2 = 4.85$, $df = 1$, $p < .05$) or experimental S + C birds ($\chi^2 = 9.93$, $df = 1$, $p < .01$). For comparison, the range of note duration in sibling control songs was 10–141 ms.

Comparison within Individuals Pre- and Posttreatment. The abnormal song elements emerged with recovery from the C treatment. Prior to C paralysis, songs of P + C birds did not have a higher proportion

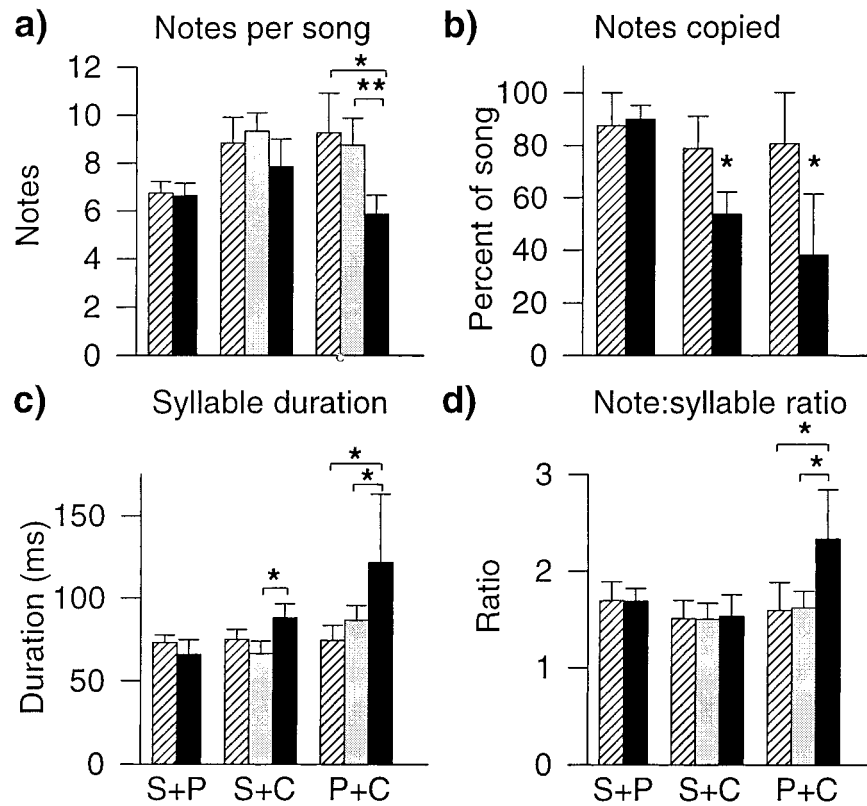


Figure 5 Song measurements of birds which received two BoTX treatments. There are no differences between experimental birds (black bars) that received a BoTX injection during both subsong and plastic song (S + P) and their control siblings (hatched bars) (unpaired t tests, $p > .05$). Postrecovery songs of experimental birds in the S + C group differ from songs of sibling controls in the number of notes copied from the tutor (b) and differ from pretreatment songs (gray bars) in syllable duration (c). Postrecovery songs of experimental birds in the P + C group differ from pretreatment songs as well as songs of sibling controls in all parameters measured [$*p < .05$, $**p < .01$; posttreatment and control comparisons, unpaired t tests; post- and pretreatment comparisons, paired t tests; (c) P + C comparisons, F tests].

of striated notes or multinote syllables than did songs of sibling controls ($\chi^2 = 1.11$, $df = 1$, $p = .293$).

A characteristically abnormal temporal pattern was evident in songs of birds in this group. This appeared to be due to increased variance of syllable duration and decreased intersyllable intervals (e.g., Fig. 8) as well as to deletions of intersyllable intervals following recovery from the C treatment. The posttreatment appearance of abnormal long-duration song elements did not significantly increase the mean syllable duration (from 86.72 ± 9.02 ms before C treatment to 121.79 ± 41.29 after recovery, Wilcoxon matched-pairs signed-ranks test), but did result in a significantly greater variance in syllable duration than that of pre-C treatment songs, and that of control siblings (Levene's test for homogeneity of variances: experimental post-C treatment compared with pre-C treatment, $p = .031$; experimental post-C treatment compared with controls, $p = .037$) (Fig. 5). Post-

recovery, these birds also had a lower mean intersyllable interval (29.1 ± 6.89 ms) compared with sibling controls (37.5 ± 4.12 ms) (two-tailed unpaired t test, $p = .02$).

The temporal pattern of song was further altered by deletions of intersyllable intervals and syllables. P + C birds collectively produced 31 syllables prior to C treatment. Of these, 13 syllables (42%) were deleted following reversal of paralysis. Nine of the 13 syllables (69%) were deleted with a concurrent loss of the adjacent intersyllable interval. Furthermore, the four deleted syllables which did not cause a change in temporal pattern were either the first or last syllables in a song motif, and thus a change in intersyllabic interval would not have been apparent. In addition, two birds dropped introductory syllables preceding songs and between motifs from their pretreatment repertoire, resulting in abnormal song organization.

Table 2 Developmental and Postparalysis Song Changes (Mean \pm S.E. % of Song)*

Treatment Group	Notes Added	Notes Deleted	Notes Changed
Subsong (S)			
Control	0.00	0.00	3.13 (3.13)
Treatment	0.00	0.00	3.68 (6.65)
Plastic song (P)			
Control	0.00	0.00	4.76 (4.76)
Treatment	0.00	0.00	7.17 (5.42)
Subsong + plastic song (S + P)			
Control	0.00	0.00	3.57 (3.57)
Treatment	0.00	0.00	3.47 (2.33)
Crystallization (C)			
Control [†]	0.00	0.00	5.56 (7.86)
Treatment	20.70 (6.83)	17.05 (6.34)	14.98 (5.74)[‡]
Subsong + crystallization (S + C)			
Control	0.00	0.00	7.30 (4.64)
Treatment	9.98 (4.61)	22.78 (11.18)	35.36 (9.65)[‡]
Plastic song + crystallization (P + C)			
Control	0.00	0.00	9.13 (4.64)
Treatment	0.00	42.56 (9.75)	7.14 (14.29)
Adult treatment			
Single	0.96 (0.96)	0.00	3.16 (2.32)
Double	1.82 (1.82)	0.00	0.00

* Song changes between ages 78 and 110 days (pre- and postcrystallization) are presented for the juvenile treatment and control groups. Groups S, P, and S + P do not experience an intervening BoTX treatment, and groups C, S + C, and P + C do sustain a period of paralysis within this time (treatment at period C). Adult treatment groups experienced one or two BoTX treatments in adulthood, at ages older than 90 days.

[†] Comparisons between experimental and control birds are conducted with unpaired *t* tests, except when the control mean is equal to zero, in which case variances cannot be compared.

[‡] Significance at $p < .05$. Bold indicates differences between experimental and control birds.

Table 3 Changes in Note Types between Days 78 and 110 (Mean \pm S.E. % Song)

Treatment Group	Note Type				
	HS	ST	CL	FM	OTH
S control	1.82 (1.82)	0.00	0.00	-1.82 (1.82)	0.00
S treatment	1.08 (2.90)	0.00	0.00	-2.38 (2.38)	1.30 (1.30)
P control	0.00	-0.65 (0.65)	0.65 (0.65)	0.00	0.00
P treatment	0.00	-7.17 (5.42)	1.67 (1.67)	0.00	5.50 (5.50)
S + P control	-4.17 (4.17)	0.00	0.00	0.00	4.17 (4.17)
S + P treatment	2.38 (2.38)	-1.59 (1.59)	1.59 (1.59)	-2.38 (2.38)	0.00
C control	5.55 (5.55)	0.00	0.00	0.00	-5.55 (5.55)
C treatment	10.29 (7.14)	-1.23 (6.48)	-7.00 (3.41)	3.20 (2.37)	-5.26 (2.62)
S + C control	-5.08 (3.15)	-2.22 (2.22)	2.22 (2.22)	2.22 (2.22)	2.86 (2.86)
S + C treatment	17.35 (8.77)	-3.10 (7.47)	-6.25 (6.25)	-4.63 (11.33)	-3.87 (3.89)
P + C control	-8.47 (4.33)	-3.70 (3.70)	3.70 (3.70)	3.70 (3.70)	4.77 (4.77)
P + C treatment	-9.00 (5.00)	13.05 (9.81)	2.08 (2.08)	-0.50 (10.14)	-5.65 (3.48)

HS = harmonic stack; ST = striated; CL = click; FM = frequency modulated; OTH = other. The acoustic properties of note-type categories are described in Materials and Methods.

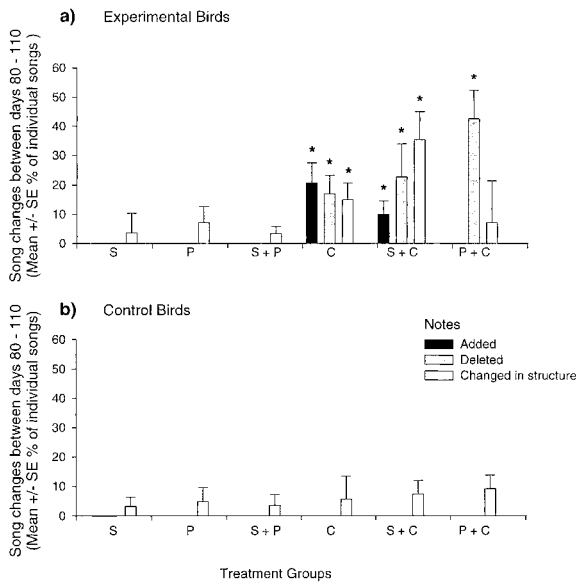


Figure 6 Types of song changes (note additions, note deletions, changes in note acoustic structure) between days 78 and 110 in (a) experimental and (b) control birds. Changes in the songs of birds in groups C, S + C, and P + C are shown prior to and following the C treatment. Songs of birds in groups S, P, and S + P are recorded over the same age interval, but do not include an intervening period of syringeal paralysis. Song changes (additions, deletions, and changes in structure) are permanent postcrystallization following recovery from BoTX. The mean number of notes per song that are changed in each individual song are calculated, rather than the number of birds which manifest song changes (i.e., changes in five notes of a 10-note song equals a change of 50%). The y axis is the mean percent change per individual for each group. Note additions occur only in groups C and S + C, and not in their sibling controls. Note deletions occur in the three groups with a C treatment, and not in their sibling controls. There is a significantly greater percent change in note acoustic structure in groups C and S + C compared with their respective sibling controls (t test, $p < .05$).

Calls

Syringeal BoTX treatments produced a characteristic change in the acoustic structure of all vocalizations; however, aberrant vocalizations which persisted after the reversal of syringeal paralysis were present only in

song elements and not in putatively nonlearned call notes (Fig. 9).

Timing of Crystallization

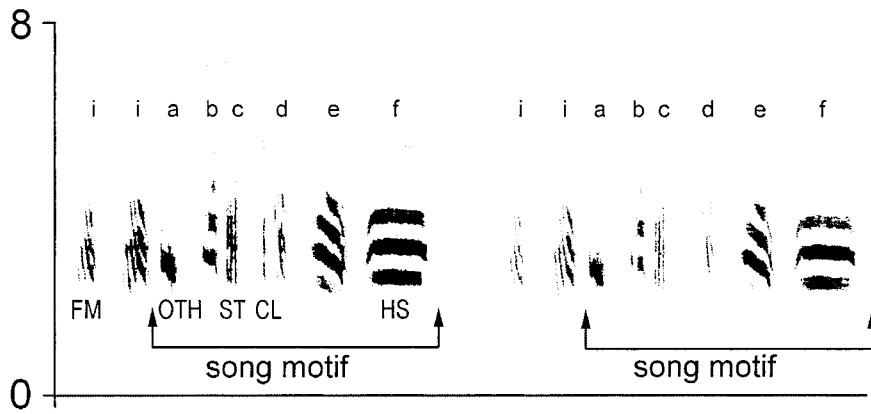
Age at crystallization was assessed only in birds without crystallization-period paralysis (groups S, P, and S + P). In each of these groups, songs crystallized at the same age as in the nontreated siblings. Between late plastic song and the end of crystallization (days 78–110), no notes were added or deleted in any of the experimental groups or their controls. Experimental and control birds exhibited changes in note structure over this time, although there were no differences among the groups in the percentages of the songs which changed (Table 2). All experimental birds had stereotyped songs by day 90 in group S + P, 91 in group S, and 93 in group P, after which there were no further changes in note structure or intersyllable intervals. Their sibling control groups achieved crystallization by days 91, 90, and 90, respectively (Fig. 10). Thus, juveniles were apparently able to compensate for the effects of BoTX administered in early periods of song motor practice without extending the normal period of song development. The timing of crystallization could not be determined precisely in groups given a BoTX treatment immediately prior to the usual age of song crystallization (C, S + C, and P + C), since changes in vocal structure due to the gradual reversal of paralysis obscured the song stereotypy indicating crystallization. However, songs were already crystallized at the time of recovery from BoTX, by day 110. Thus, if crystallization were delayed, it would be only by a few days. All posttreatment songs were stable, remaining invariant for at least 9 months, after which time the birds were no longer recorded.

Neuromotor Substrates

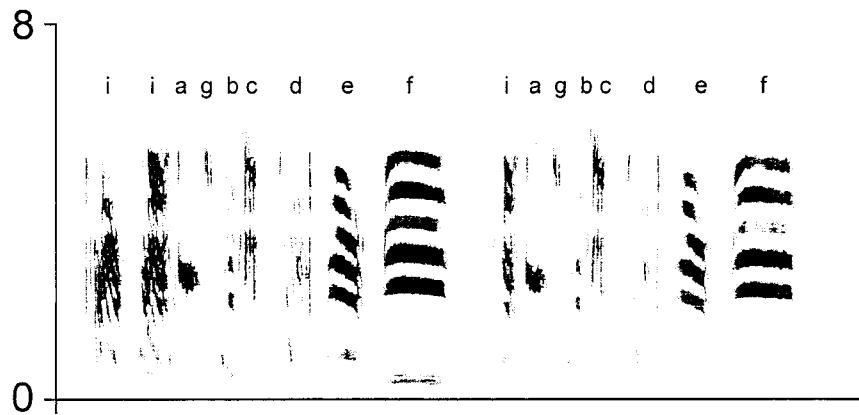
Syringeal muscle size of two birds (one from group S + C and one from P + C) with the most severe adult song aberrations was indistinguishable from that of three control adult birds, and clearly larger than the atrophied muscles of five other adults that were syr-

Figure 7 Spectrographs of the song of the tutor (a) and an experimental offspring in the S + C group following a BoTX injection at day 25, prior to the C injection at day 79. Shown here at day 78 (b) and following recovery from the C treatment, at day 138 (c). At day 78, the song is similar to that of the tutor, showing no effect of the S period BoTX treatment. After recovery from the C treatment, the temporal pattern of the song is changed, and acoustic structure of notes is altered. New notes are indicated by numbers. The tutor's song contains notes in the acoustic categories: FM = frequency modulated; OTH = other; ST = striated; CL = click; and HS = harmonic stack.

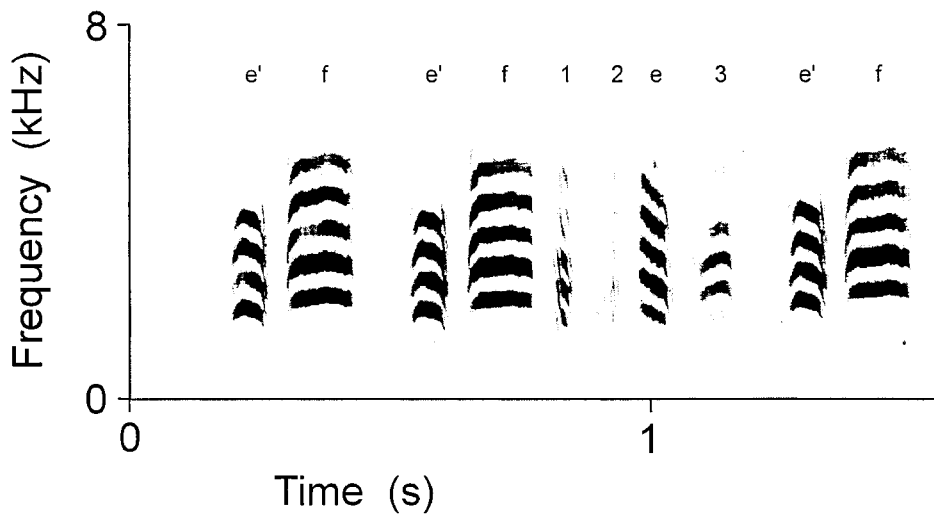
a) Tutor



b) Experimental bird 1: day 78, after subsong treatment



c) Experimental bird 1: day 138, after subsong and crystallization treatments



ingely denervated for comparison by bilateral section of the tracheosyringeal branch of the hypoglossal nerve (NXIIIs) at approximately day 40. There were no differences between experimental and control birds in the estimated nXII volume, density, total neuron number, or number of BHRP-labeled motoneurons (two-way unpaired t test, $p > .05$) (Table 4). Motor end plates in the syringeal muscles of three experimental birds from group P + C, stained by injecting cholera toxin B into NXIIIs at age 3 years, 6 months (i.e., slightly more than 3 years, 3 months after the second BoTX injection), appeared superficially similar in the light microscope to those of similarly injected control birds (J. M. Wild, personal communication). The songs of these birds were still abnormal at the time of the cholera toxin B injections.

DISCUSSION

Irreversible Abnormalities in Song Development

The absence in call notes of abnormalities apparent in song elements suggests that temporary paralysis selectively affects the acquisition or production of learned, rather than nonlearned, vocalizations. Furthermore, within an aberrant song, some notes may be excellent copies of the tutor's notes, while others may be structurally abnormal. Thus interference with syringeal function at particular times impairs specific song elements rather than having a global effect on the entire vocalization. However, the mechanism underlying these developmental abnormalities, whether central, peripheral, or both, remains to be determined. The extent to which lasting deficits in song are due to inappropriate auditory or syringeal feedback at critical phases in song development is also unknown.

Sensitive Periods in Song Learning: Sensory Phase and Sensorimotor Phase

The sensory phase, during which a memory of the tutor's song is formed, is restricted to a sensitive period in development. In zebra finches, a tutor's song may be partially encoded in memory as early as day 25 (Immelman, 1969) and completely copied by day 35 (Bohner, 1990). A tutor's influence on juvenile

song may decrease sharply at about day 65 (Eales, 1985) but perhaps not terminate completely until sometime between days 66 and 80 (Immelman, 1969). In a normal rearing environment, exposure to a tutor before or after these ages has no influence on the song that is learned (Immelman, 1969; Arnold, 1975). Furthermore, juveniles removed from their father and housed with a second tutor at either day 35, 50, or 65 reveal that most song memorization occurs between days 50 and 65 (Eales, 1985). Thus, within the sensitive period, this is a time of heightened receptivity to a tutor's song. Similarly, in the present study, we determined that within the sensorimotor integration phase, there is a period of increased sensitivity to motor disturbance (Table 5). Furthermore, in both the sensory and sensorimotor phases, experience encountered late in development appears to have a greater influence on the outcome of song production than does experience during early stages of song development.

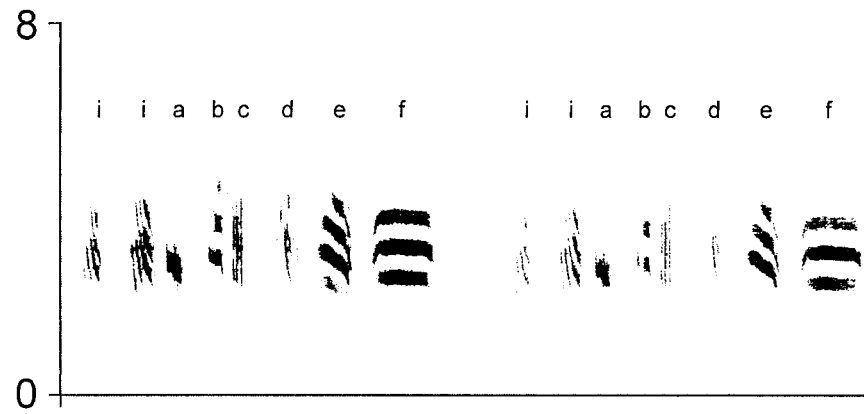
Relative Contribution of Single Song Stages

Comparison of S, P, and C Groups. Interference with syringeal function during crystallization (group C) has an irreversible effect on song production. Whereas the acoustic structure and temporal pattern of adult songs of these birds are species typical, within-individual comparisons of songs before and after treatment reveal that after the reversal of paralysis, songs are not identical to their pretreatment form, but contain permanent changes (note additions, deletions, and changes in acoustic structure) which do not occur in sibling controls during the same age interval. Thus, a temporary loss of motor control during the terminal stage of song acquisition results in permanent changes in the nearly crystallized motor pattern. However, these changes do not include aberrant or species-atypical acoustic structure or temporal patterning. The properties of the altered songs are still within the range of variation present in songs of nonexperimental adult zebra finches.

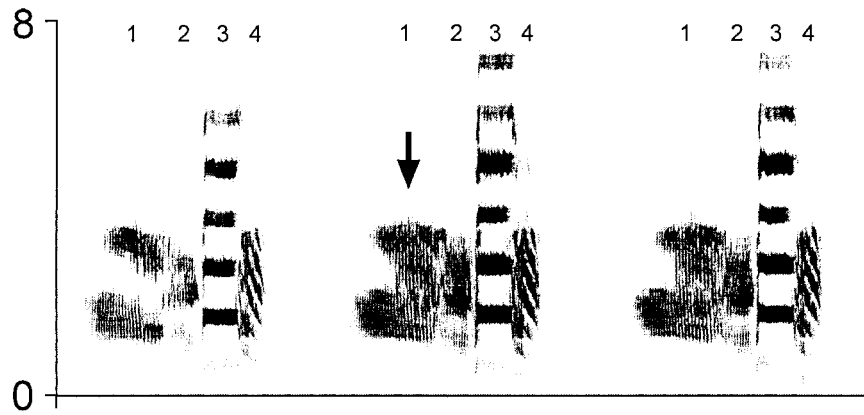
The relative contributions that subsong, plastic song, or the period of song crystallization make to song development cannot be deduced by comparing the songs of birds in these experimental groups (S, P,

Figure 8 Spectrographs of the song of the tutor [the same bird as in Fig. 7(a)], an experimental offspring in group P + C following recovery from the C treatment (injection at day 83) shown at day 138 (b), and a control sibling shown at day 130 (c). The adult songs of birds in group P + C characteristically contain abnormally long fricatives (arrow) and multinote syllables, and are produced in an abnormal temporal pattern.

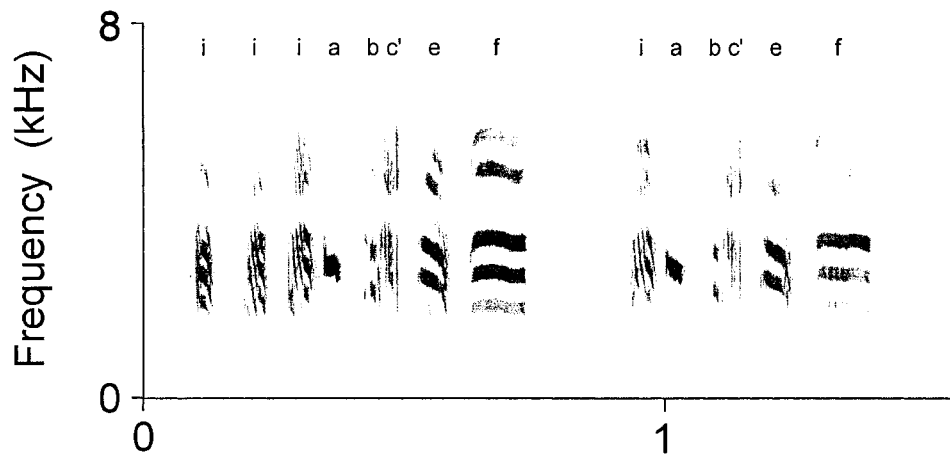
a) Tutor



b) Experimental bird 1



c) Control bird 2



or C) with each other, or with those of their sibling controls. This is because none of these treatments produced temporal or structural changes that were abnormal for this species. Within-individual pre- and posttreatment song comparisons can directly reveal specific changes following paralysis, but can only be conducted on birds in group C whose pretreatment song has stable features.

The absence of an overt effect on adult song of BoTX treatment during subsong and/or plastic song raises important questions regarding the role these developmental stages play in song acquisition and the relationship between these stages and song crystallization. The birds in group S + P developed normal adult songs without a delay in the timing of crystallization, even though their vocal production was disrupted during 40–80% of their sub- and plastic song stages. This suggests that maturational processes may have a larger influence on juvenile song development than is generally recognized, and/or that a relatively short period of motor learning is sufficient to match song elements to those of the tutor.

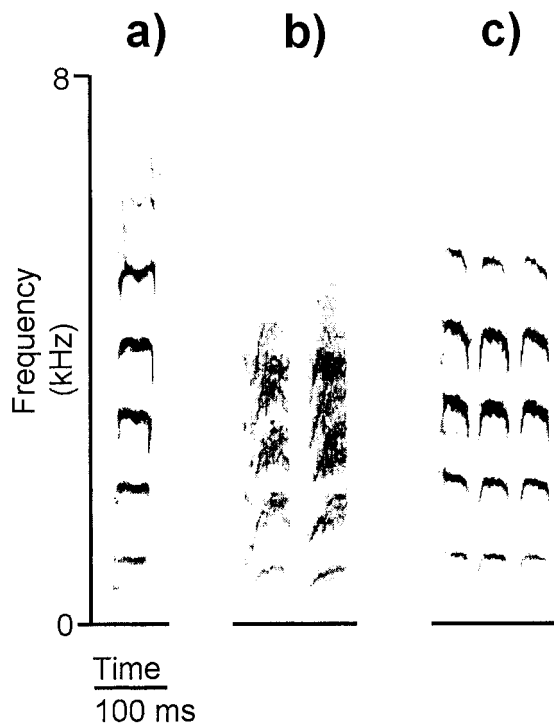


Figure 9 BoTX treatments during song development have only temporary effects on nonlearned vocalizations. The nonlearned call note of an individual in group P + C which received a BoTX injection at age 55 days is shown here on day 82, 1 day before the second BoTX treatment (a). The same call note 6 days after the second BoTX treatment has a noisy acoustic structure (b). Unlike learned vocalizations, nonlearned call notes recover their preoperative structure following reversal of syringeal paralysis [day 138 (c)].

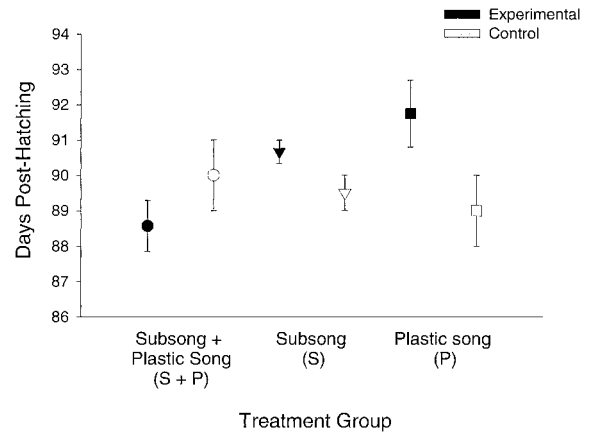


Figure 10 Ages at which song crystallization is completed. The mean (\pm S.E.) ages at which the birds in each treatment group and their respective control groups achieve a stereotyped song with no further changes in acoustic structure or intersyllable intervals. None of the differences between experimental birds and sibling controls are significant (unpaired t test, $p > .05$). The difference in mean crystallization age among the three treatment groups combined, and the three control groups combined, is also not significant (not shown: treatment mean, 89.9 ± 0.58 ; control mean, 89.5 ± 0.43 ; unpaired t test, $p > .05$).

Early Stages of Motor Practice Contribute Differentially to Song Development

Comparison of S + C and P + C Groups. Although disruption of subsong and plastic song does not produce detectable abnormalities in adult song production, we propose that vocal motor practice during both these stages does contribute to the development of the song motor pattern. This is revealed by the differences in the types of defects that are characteristic of the S + C and P + C groups. S + C songs have a normal temporal pattern and syllable duration. However, these songs vary little in acoustic structure since they are mostly composed of harmonic stacks. The songs of the P + C group have an abnormal temporal pattern and are composed mainly of multinote syllables and long-duration, broadband, striated notes. The differences in aberrations in the songs produced by S + C and P + C birds are not due to variations in tutoring, as each S + C bird has a P + C sibling, and each sibling pair was tutored by their mutual father. The contrasts between the two groups must therefore be due to the timing of the early period of motor impairment. The different effects of paralysis during these stages suggests that different developmental processes occur during subsong and plastic song. Paralysis may interfere with the emergence of individual syringeal motor patterns, the coordination between syringeal

Table 4 Measurements of Experimental and Control nXII (Mean \pm S.E., Left and Right Nuclei Combined)

Treatment	nXII Volume (mm ³)	Neuronal Density (cell/mm ³)	Estimated Total Neuron Number	% Neurons Labeled
Experimental	0.0916 (127.89)	19,714.00 (491.34)	1817.75 (285.80)	50.01 (1.56)
Control	0.1025 (146.98)	18,991.83 (1281.33)	1856.33 (161.65)	50.63 (8.05)

and respiratory motor patterns, and/or with the sequential assemblage of component motor patterns.

The evidence for sequential acquisition of component motor patterns parallels Immelman's (1969) observations of sensory learning. He determined that various characteristics of a song model are memorized sequentially at different ages within the sensory phase of song learning. Immelman transferred zebra finch eggs to Bengalese finch clutches and then removed the juvenile zebra finches from their adopted tutor and placed them in isolation at various ages. Zebra finches that were isolated before day 40 developed songs with notes derived from the tutor but which differed in sequence and phrase length. Birds isolated from their tutor between days 38 and 66 produced notes that were identical to those of the tutor, but the songs differed in sequence and phrase length. Birds isolated after day 80 copied structural as well as temporal components from their tutor, including syllables, sequence, phrase length, and number of syllables. Immelman concluded that the acoustic structure of song elements is memorized first, and that this could begin as early as day 25 but is not complete until day 38. Note sequence, song length, and temporal components are memorized later, after juvenile song production has begun, during days 66 and 80.

Following the crystallization-period BoTX treatment, songs of birds with a previous period of motor disruption during subsong have a normal temporal pattern but lack variation and complexity in acoustic structure. In contrast, crystallization-period paralysis subsequent to an earlier plastic song treatment results in songs with some acoustic variation but containing aberrant temporal characteristics. It appears that the

sequence of song characteristics acquired during song motor development may reflect the order of component characteristics memorized during the sensory phase: acoustic structure, followed by note order and temporal patterning.

Song Abnormalities Are Evident Only When Motor Disruption Includes the Sensitive Period during Song Crystallization

Comparison of S + C, P + C, and S + P Groups. A between-group comparison of the adult songs of groups with two periods of paralysis suggests that the time around song crystallization is a critical sensorimotor period in vocal development. Our data are not consistent with the alternative hypothesis that young birds simply accumulate sensorimotor experience with time and therefore lose less when vocal production is disrupted early in song development than when the disruption occurs late in development. This model predicts a progression of increasing song impairment in the treatment group sequence: S, P, S + P, C, S + C, P + C. We do not find such a pattern, however, since treatments at stages S, P, and S + P have no detectable effect on song motor development. Neither are the data compatible with the hypothesis that neural plasticity is depleted by each injection of BoTX in a way that is independent of the developmental stage: i.e. that enough plasticity remains to recover normal song after a single treatment but not after two treatments. This hypothesis fails to account for the absence of a detectable effect on adult song of the S + P group. The development of abnormal song in both S

Table 5 Treatment Groups Compared with Their Respective Control Siblings Either in Adult Song Production (Column 2) or during Development (Columns 3 and 4)

Treatment Group	Adult Song Structure	Within-Individual Song Changes	Timing of Crystallization
Subsong (S)	Normal	*	Normal
Plastic song (P)	Normal	*	Normal
Subsong + plastic song (S + P)	Normal	*	Normal
Crystallization (C)	Normal	Slight	*
Subsong + crystallization (S + C)	Abnormal	Large	*
Plastic song + crystallization (P + C)	Abnormal	Large	*

* Cannot be measured.

+ C and P + C groups is in striking contrast to the development of normal song by group S + P, and strongly suggests the existence of a period of heightened sensitivity to motor disruption during song crystallization.

Song development in zebra finches is accompanied by both age- and experience-related changes in gene expression, some of which are hypothesized to define critical periods (e.g., Clayton, 1997). Activity-dependent developmental processes may therefore be disrupted by insufficient or abnormal activity during paralysis, resulting in changes at central synapses in the song control system, in sensory feedback pathways, and/or at the neuromuscular junction. In the early stages of subsong and plastic song, the residual effects of paralysis are apparently masked, providing this initial insult is not followed by a second paralysis at the time of song crystallization. However, although there is no apparent behavioral consequence of early sensorimotor disruption, paralysis may diminish the potential for reorganization necessary to recover from a second insult if this insult occurs during a sensitive period. The heightened sensitivity to sensorimotor disruption during song crystallization may reflect a developmental modulation in neural plasticity as the song motor pattern becomes established, perhaps corresponding to the consolidation of synaptic connections, committing neurons to various motor circuits for song production. Sensitivity to disruption at this time may also reflect a period of increased dependence on motor control or sensorimotor feedback necessary to establish the integrity of the motor circuits. Once established, however, the adult motor pattern is no longer sensitive to temporary sensorimotor disruption.

A similar relationship between the timing of disturbance during motor development and the resulting adult motor pattern is evident in the development of quadrupedal locomotion. The effects of a prolonged period of aberrant sensory feedback, beginning at the onset of quadrupedal walking and persisting for the duration of the developmental period, are reversed if normal feedback is restored before the end of maturation (comparable to the S + P treatment). However, if abnormal sensory feedback persists through the transition from juvenile to adult walking patterns, permanent motor abnormalities result (comparable to the S + C and P + C groups) (Altman et al., 1975; Walton et al., 1992).

Parallels between developmental requirements of speech and birdsong have led to the speculation that there may be similar processes underlying the acquisition of learned vocal motor behaviors in both taxa (Marler, 1970; Marler et al., 1981). In light of this suggestion, it is noteworthy that human infants with

long-term prelinguistic tracheostomies, preventing vocalization during babbling, show more rapid recovery from a lack of babbling early in vocal development than later, at the onset of word production (Simon et al., 1983; Locke et al., 1990; Kamen et al., 1991). The relatively late period of increased sensitivity to perturbations of motor practice may thus be a general principle in the development of motor patterns, perhaps due to age- or experience-dependent limitations in plasticity of associations between motor commands and sensory feedback.

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