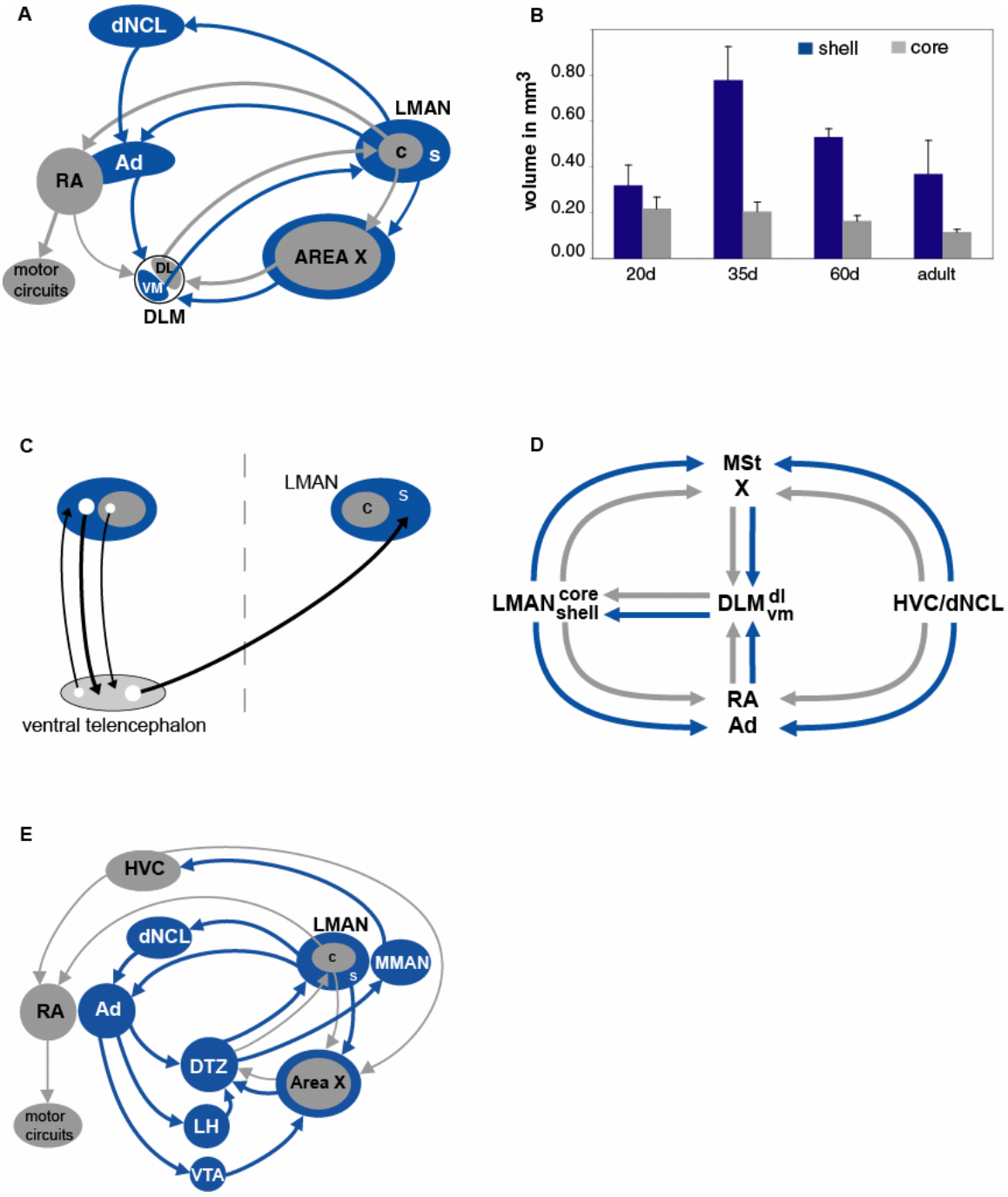
Parallel Pathways for vocal learning in basal ganglia of songbirds

Sarah W. Bottjer and Brie Altenau

Supplementary Figure S1



Supplementary Figure S1. Hodological and morphological aspects of $LMAN_{shell}$ circuits. (**A**) **Major axonal connections made by core and shell regions of LMAN**. The cortical nucleus LMAN is a unitary structure, but is composed of separate core and shell subregions which give rise to independent parallel pathways that traverse the basal ganglia and thalamus[1-8]. The core region contains a high density of magnocellular neurons, and the surrounding shell region contains both magnocellular and parvocellular neurons. LMAN provides the output of a basal ganglia pathway from Area X (and surrounding striatum) to DLM (in dorsal thalamus) to LMAN. Area X and surrounding striatum contain both striatal and pallidal neurons. $LMAN_{core}$ (gray) projects to RA within the area of avian brain that is analogous to motor cortex; RA projects to descending motor circuits that activate vocal and respiratory muscles. In contrast, $LMAN_{shell}$ (blue) projects to Ad, an area of motor cortex adjacent to RA. Ad does not make direct connections to hindbrain vocal-motor circuits, but makes a variety of axonal projections, including a projection to DLM that forms a recurrent loop from $LMAN_{shell}$→Ad→DLM_{VM}→$LMAN_{shell}$. $LMAN_{shell}$ also projects strongly to a specific region within dNCL, a cortical polymodal association region similar to a region of chick brain that is involved in imprinting[9, 10]. The act of producing song strongly induces expression of the immediate early gene product ZENK in dNCL of juvenile birds that are listening to tutor song (Bottjer, Alderete, and Chang, unpublished data), suggesting a possible role for shell circuitry in auditory-motor integration or motor learning. In zebra finches, dNCL projects to Ad, such that $LMAN_{shell}$ projects directly to Ad and indirectly via dNCL. Lesions of Ad remove both the direct target of $LMAN_{shell}$ neurons, as well as that of the polysynaptic pathway from $LMAN_{shell}$→dNCL→Ad. Separate groups of neurons in DLM (DL, dorsolateral versus VM, ventromedial) project to either core or shell, respectively. Core and shell regions of LMAN also project to Area X and surrounding medial striatum, respectively, to form additional recurrent loops. Not shown here for the sake of clarity is a projection from Ad to DMP (part of a dorsal thalamic zone, DTZ, that includes both DLM and DMP); DMP projects to MMAN, which projects in turn to HVC (see E). The dorsal cap of RA makes a minor projection to the dorsal thalamic zone, including DLM[8, 11].

Abbreviations: LMAN (lateral magnocellular nucleus of the anterior nidopallium; c = core region; s = shell region), Area X (Area X of the medial striatum), RA (robust nucleus of the arcopallium), Ad (dorsal arcopallium), DLM (medial dorsolateral nucleus of the thalamus; DLM_{DL} contains neurons in the dorsolateral portion of DLM that project to $LMAN_{core}$ whereas DLM_{VM} contains neurons in the ventromedial portion of DLM that project to $LMAN_{shell}$), dNCL (dorsal region of the caudolateral nidopallium), the blue region surrounding Area X is medial striatum (MSt).

(**B**) **Changes in the volume encompassed by core (gray) and shell (blue) regions of LMAN.** The volume of $LMAN_{core}$ decreases slightly despite maintaining a fixed number of projection neurons[12]. The volume of $LMAN_{shell}$ exhibits pronounced growth during early stages of vocal learning (20-35 days of age), followed by substantial regression between 35-40 days and adulthood[1, 4]. This pattern suggests that $LMAN_{shell}$ makes some unique contribution to mechanisms of vocal learning during development when its volume is maximal, which could account in part for the enhanced capacity for vocal imitation during the sensitive period. The dramatic increase in overall volume of $LMAN_{shell}$ is accompanied by pronounced regression of individual thalamic axon arbors (from DLM) within this region[13], indicating substantial refinement in neural connectivity within the shell pathway during vocal development. Thus the capacity to carry out learning-related functions may be enhanced in juvenile birds when the size of $LMAN_{shell}$ is greatly enlarged and presumably the information-processing capacity of shell circuitry is increased.

(**C**) **Ipsi- and contra-lateral projections connect left and right core and shell regions**. $LMAN_{shell}$ makes a strong ipsilateral projection through the basal forebrain (ventral arcopallium) to contralateral $LMAN_{shell}$, and this projection overlaps with an ipsilateral projection from $LMAN_{core}$ to the same region of basal forebrain as well as with a reciprocal projection from basal forebrain back to $LMAN_{shell}$[4]. The robust contralateral projection between left and right $LMAN_{shell}$ and its overlap

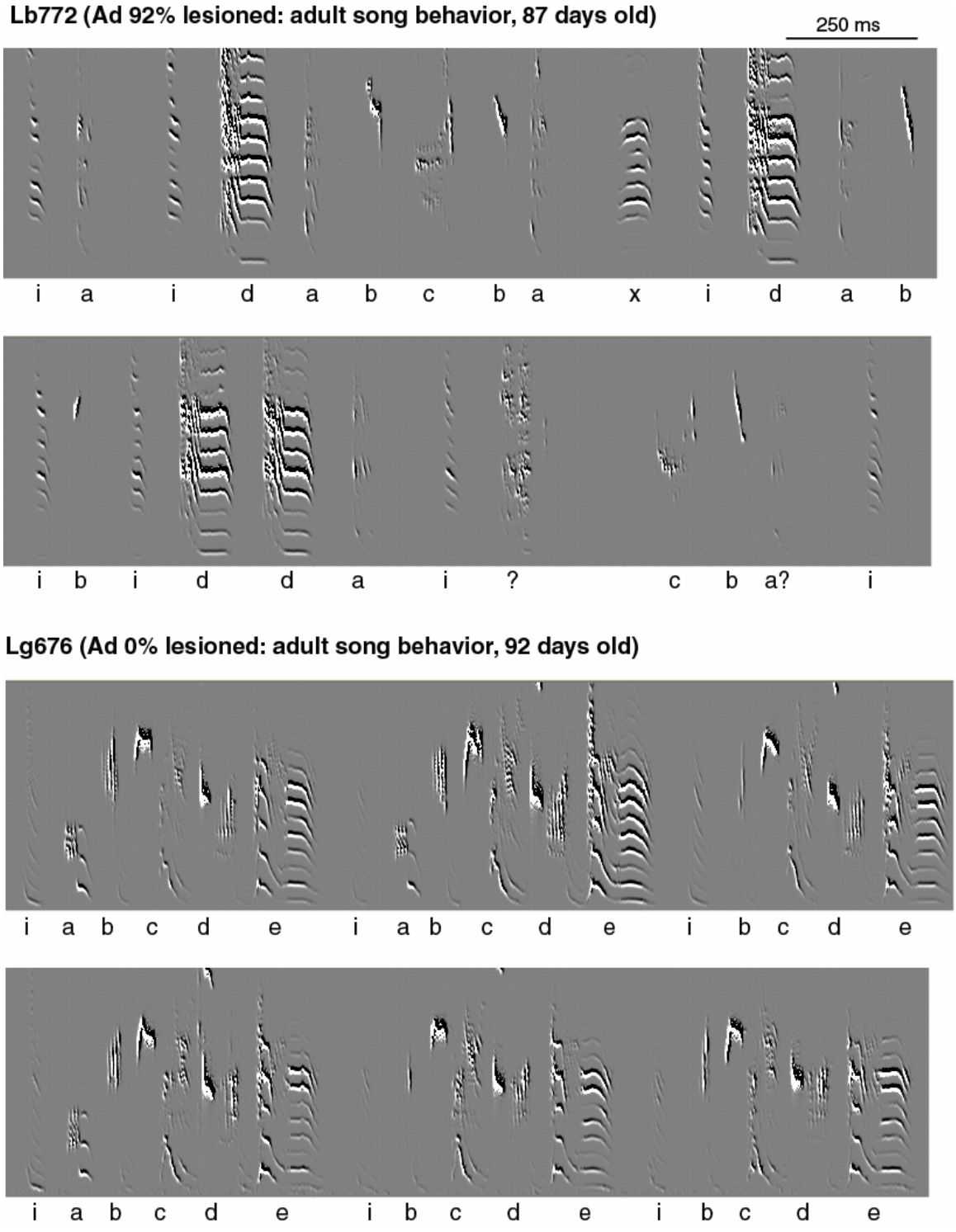
with a projection from $LMAN_{core}$ suggests a role in inter-hemispheric coordination during acquisition of novel vocal motor patterns and integration of information between core and shell pathways.

(**D**) **Multiple points of possible convergence between core and shell pathways**. The connections of LMAN shell (blue) and core (gray) pathways may integrate information between them through several points of overlap, including DLM which receives a strong projection from Ad and a modest projection from the dorsal region of RA, as well as parallel projections from Area X and medial striatum (MSt)[6, 8, 11, 14, 15]. Because Ad appears to project throughout DLM (as well as to DMP), information processed in both core and shell pathways may converge in this dorsal thalamic zone[3, 6, 7]. An interesting contrast is that HVC sends separate projections to RA and X, whereas core and shell each send single (collateral) projections to RA/X and Ad/MSt (medial striatum), respectively. Not shown here (for clarity) is the direct projection from $LMAN_{shell}$ to dNCL and from DMP→MMAN→HVC. The pathway from RA to DLM is not a major projection in adult birds, but has not been examined in juveniles. The contralateral and reciprocal projections made by core and shell regions of LMAN via the ventral forebrain (C) represent another possible source of integration between core and shell pathways, as well as a possible integration with limbic pathways. All of these pathways could serve as points of cross-talk between core and shell pathways.

(**E**) **Additional axonal projections of $LMAN_{shell}$**. Ad projects to a dorsal thalamic zone (DTZ) that includes not only DLM, which makes feedback connections to LMAN, but also DMP, which makes feedforward connections to HVC (via MMAN). In addition, Ad projects to the lateral hypothalamus (which projects in turn to DTZ) and to the ventral tegmental area that sends a dopaminergic projection to Area X. The projections of Ad to lateral hypothalamus as well as brainstem regions that produce biogenic amines suggest an involvement with neuromodulatory inputs to the song system that could contribute to mediating motivational aspects of song and changes in song that occur as a function of different behavioral states and social contexts[16-19]. See [3, 6-8] for additional details. As indicated above (C, D), an important question regards how functional integration can be achieved across parallel basal ganglia loops in general[20, 21]. The organization of core and shell pathways of LMAN offer multiple opportunities for both spatial and temporal convergence[3, 22], suggesting that information processing in shell circuitry could provide instructive inputs to other pathways, including HVC and $LMAN_{core}$, that are necessary for changes in vocal motor output[15, 23, 24].

Abbreviations: LMAN (lateral magnocellular nucleus of the anterior nidopallium; c = core region; s = shell region), MMAN (medial magnocellular nucleus of the anterior nidopallium), Area X (Area X of the medial striatum), HVC (high vocal center), RA (robust nucleus of the arcopallium), Ad (dorsal arcopallium), DTZ [dorsal thalamic zone; includes both DMP (the dorsomedial nucleus of the posterior thalamus) and DLM (medial dorsolateral nucleus of the thalamus)], dNCL (dorsal region of the caudolateral nidopallium), LH (lateral hypothalamus), medial striatum (MSt; surrounds Area X), VTA (ventral tegmental area).

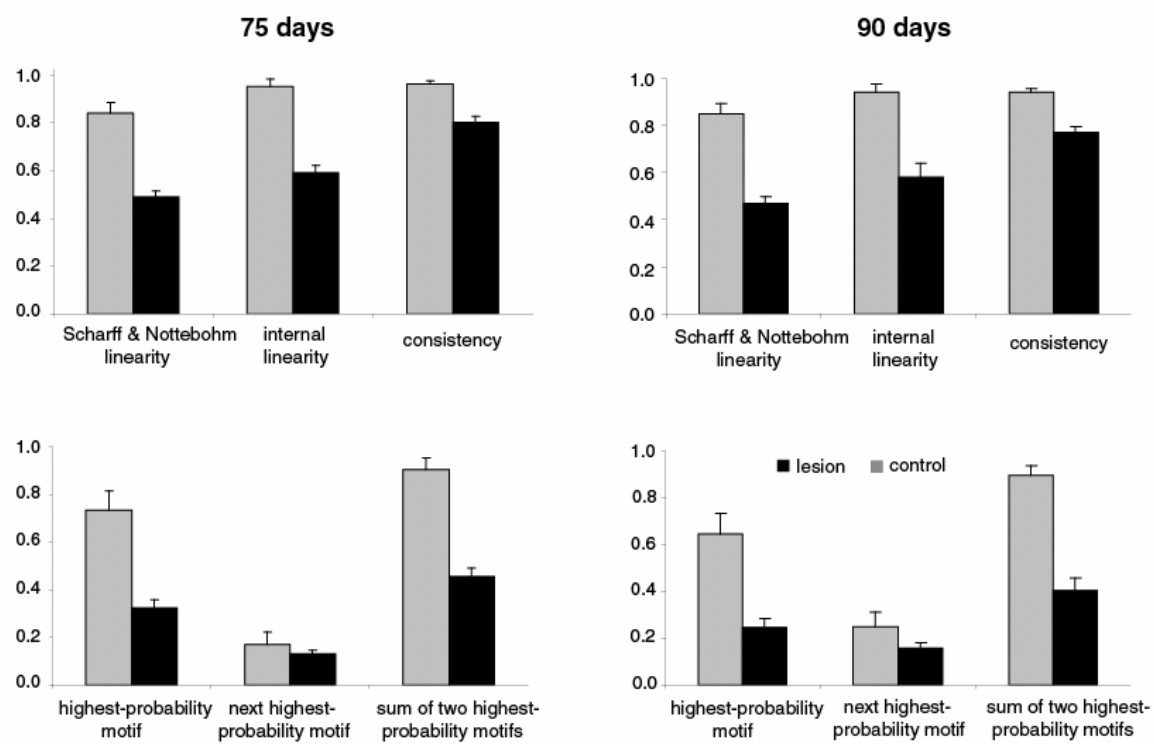
Supplementary Figure S2



Supplementary Figure S2. Disrupted vocal behavior produced by juvenile Ad lesions. Bottom two panels: sonograms showing song behavior of adult bird Lg676, which received a control lesion at 45 days of age that did not encroach on Ad. Juvenile birds that received control

surgeries or minor damage to Ad produced normal song behavior, consisting of highly stereotyped individual syllables (i.e., acoustic elements with stable harmonics and frequency modulation). As is typical of normal birds, one stable temporal sequence of syllables was repeated most often, representing the canonical song "motif" for that bird. Top two panels: sonograms showing abnormal song behavior of adult bird Lb772, which received substantial lesion damage to Ad at 44 days of age. This bird produced some call-like song syllables (**i**, **d**, **x**) along with three more complex song syllables (**a**, **b**, **c**). As in other birds with substantial damage to Ad, the morphology of individual syllables was not obviously abnormal with respect to basic phonological features, although syllabic structure appeared slightly different and more variable than in normal birds (e.g., different iterations of syllable **a** are more variable than is typical). In rare instances unrecognizable syllables were produced (e.g., the noisy syllable marked "**?**" in the second panel from top). In terms of syllable ordering, this bird produced syllables with little or no stereotypy of temporal sequence, such that it was impossible to identify a basic song motif. In addition, the number of syllables produced in each apparent motif varied considerably, such that normal parsing of song behavior into repeated motifs during a bout of song was not evident. The disrupted song behavior of birds with Ad lesions is somewhat similar to normal subsong produced by juvenile birds. Y-axis is frequency (0 to 8 kHz) and X-axis is time. Sample vocal recordings (wav files) from the birds in this figure are provided as additional Supplementary Information.

Supplementary Figure S3



Supplementary Figure S3. Quantitative measurements of temporal stereotypy in vocal behavior. Corresponding measures are shown for lesioned versus control birds at 75 days of age (left panels) and 90 days (right panels). Top panels show two different measures of degree of sequence linearity of song syllables plus a measure of consistency, which quantifies the probability of consistent syllable transitions (see Supplementary Methods). Scharff & Nottebohm linearity scores measure the number of different syllable-to-syllable transitions for each bird by calculating the number of different syllables produced divided by the number of different syllable transitions[25]. At 75 days of age, control birds produced higher linearity scores than did lesioned birds (see text). We also calculated a second linearity score called internal linearity in which syllable transitions at the ends of motifs are not counted, such that birds that produce both **abcd** and **abcde** are still treated as perfectly linear[17, 26, 27]. As expected, internal linearity scores were higher for both control and lesioned birds, since it does not penalize birds for producing partial sequences as long as each syllable-to-syllable transition is conserved. Nevertheless, this measure also showed significantly lower scores for lesioned birds compared to controls (at 75 days: 0.95 $\pm$ 0.03 for control birds versus 0.59 $\pm$ 0.03 for lesioned birds; $U = 0$, $p < 0.001$). Scores are depicted as mean $\pm$ SEM. See Supplementary Methods for additional details and Supplementary Tables S1 and S2 for individual data.

As an additional means of assessing sequence stereotypy, we calculated the syllable sequence produced on the highest proportion of total motifs as well as that produced with the second-highest probability, plus their summed occurrence (i.e., motif variants with the highest and the next-highest probability were added together to obtain the sum of the two highest-probability motifs)[28]. These two motif variants should account for most vocal utterances in normal birds with stereotyped songs (i.e., normal birds produce their canonical motif most often and may also produce a "splice variant" on some proportion of song motifs). Left bottom panel shows that

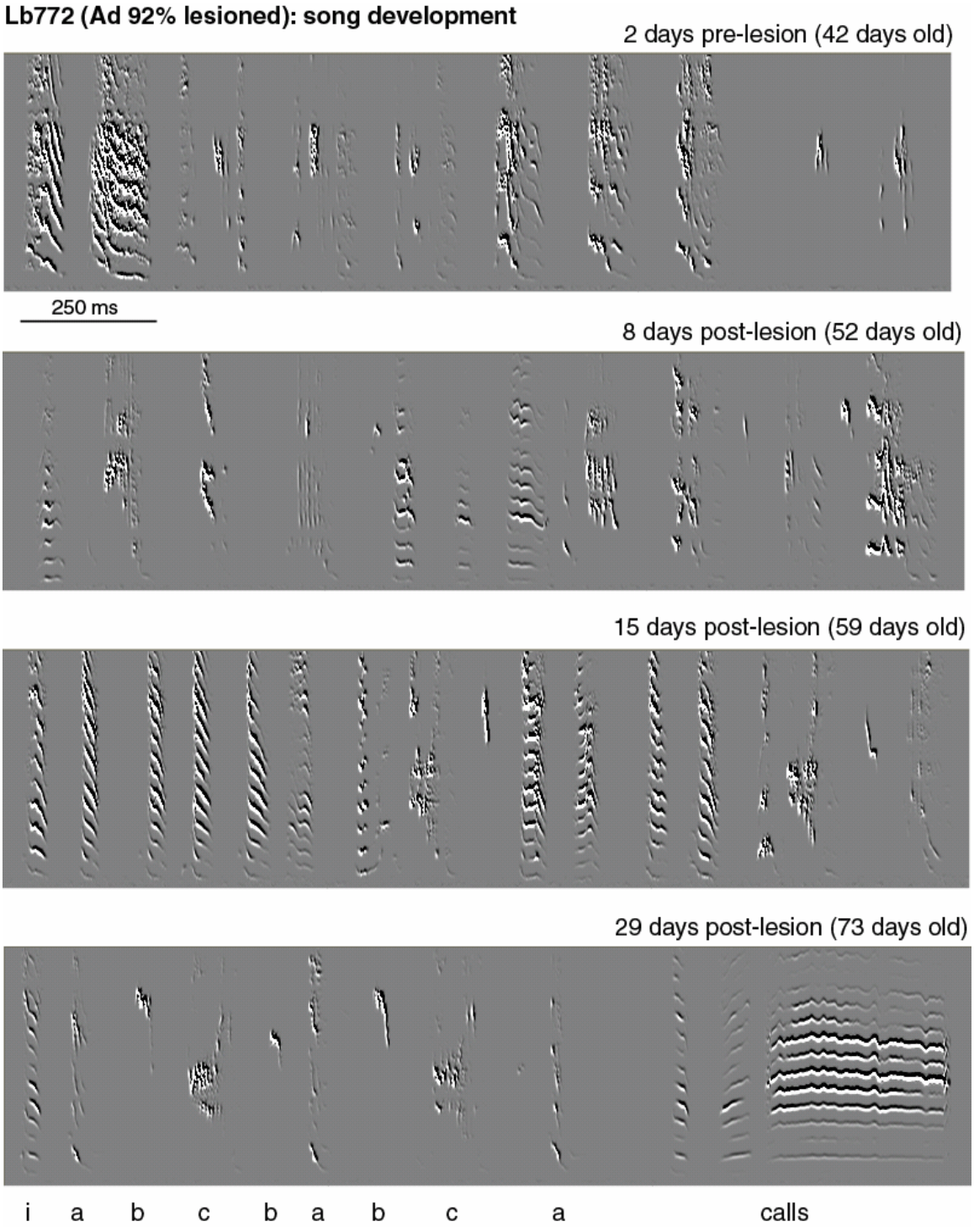
control birds at 75 days produced the highest-probability motif significantly more often than did birds with substantial lesion damage to Ad (see text). In contrast, control and lesioned birds did not differ in terms of producing the motif with the next highest probability: control birds produced an alternate motif 17% of the time whereas lesioned birds produced an alternate motif 13% of the time ($p > 0.10$). The sum of these two measures was different (90% for controls versus 45% for lesioned birds; $U = 0$, $p < 0.001$), due to the strong tendency of control birds to produce a single canonical motif most of the time. This lower level in lesioned birds is due to the fact that they produced many more different sequences overall.

In general, birds analyzed at the older age (90 days) showed the same basic pattern of disruption seen earlier at 75 days of age. No change was observed in the overall pattern of scores (Scharff & Nottebohm linearity: 0.85 ± 0.04 for controls versus 0.47 ± 0.03 for lesioned birds, $U = 0$, $p < 0.001$; internal linearity: 0.94 ± 0.04 for controls versus 0.58 ± 0.06 for lesioned birds, $U = 5.5$, $p < 0.02$; consistency: 0.94 ± 0.01 for controls versus 0.77 ± 0.03 for lesioned birds, $U = 1.5$, $p < 0.002$). In addition, control birds produced their highest-probability motif 65% of the time on average, whereas birds with substantial lesion damage to Ad produced the highest-probability motif only 25% of the time ($U = 2.5$, $p < 0.01$). As at 75 days of age, control and lesioned birds at 90 days did not differ in terms of producing the motif with the next highest probability: control birds produced an alternate (second-highest frequency) motif 25% of the time whereas lesioned birds produced an alternate motif 16% of the time ($p > 0.10$). Thus, the degree and pattern of disruption in learned vocal behavior caused by damage to Ad was established by 75 days of age and changed little or not at all thereafter. Likewise, the degree of sequence stereotypy established by control birds was mature by 75 days and did not change by the time the birds reached full adulthood (90 days).

We also tested whether the extent of lesion damage to Ad correlated with the degree of disruption in sequence stereotypy by comparing the total percent of Ad lesioned (left and right sides combined) to all three measures of linear sequence stereotypy across all birds. An inverse relation was obtained in each case such that birds with greater amounts of lesion damage had lower scores for Scharff & Nottebohm linearity, internal linearity, and consistency. A Pearson product-moment correlation yielded significant values for all three measures: Scharff & Nottebohm linearity, $r = -0.81$ ($t = 5.97$, $p < 0.001$); internal linearity, $r = -0.75$ ($t = 4.92$, $p < 0.001$); consistency, $r = -0.67$ ($t = 3.93$, $p = 0.001$). Thus, larger lesions of Ad correlate with greater disruption to the ability to produced a stereotyped vocal sequence.

These data show that Ad-lesioned birds never developed a stereotyped temporal sequence of syllables. Interestingly, inactivation of LMAN targeted to the core region acutely increases stereotypy of syllable ordering in juvenile birds from 59-72 days of age[29]. An interesting question for further investigation is whether core versus shell pathways promote variability versus stability in vocal output (cf.[25, 26]).

Supplementary Figure S4



Supplementary Figure S4. Vocal development in a juvenile bird with an Ad lesion. Sonograms show vocal behavior of Lb772 at ages ranging from 42 days (2 days prior to surgery) up to 73 days (one month following surgery). Although adult song behavior was highly abnormal (Fig. S2), no disruption of song was observed during the first several days post-surgery. The pre-

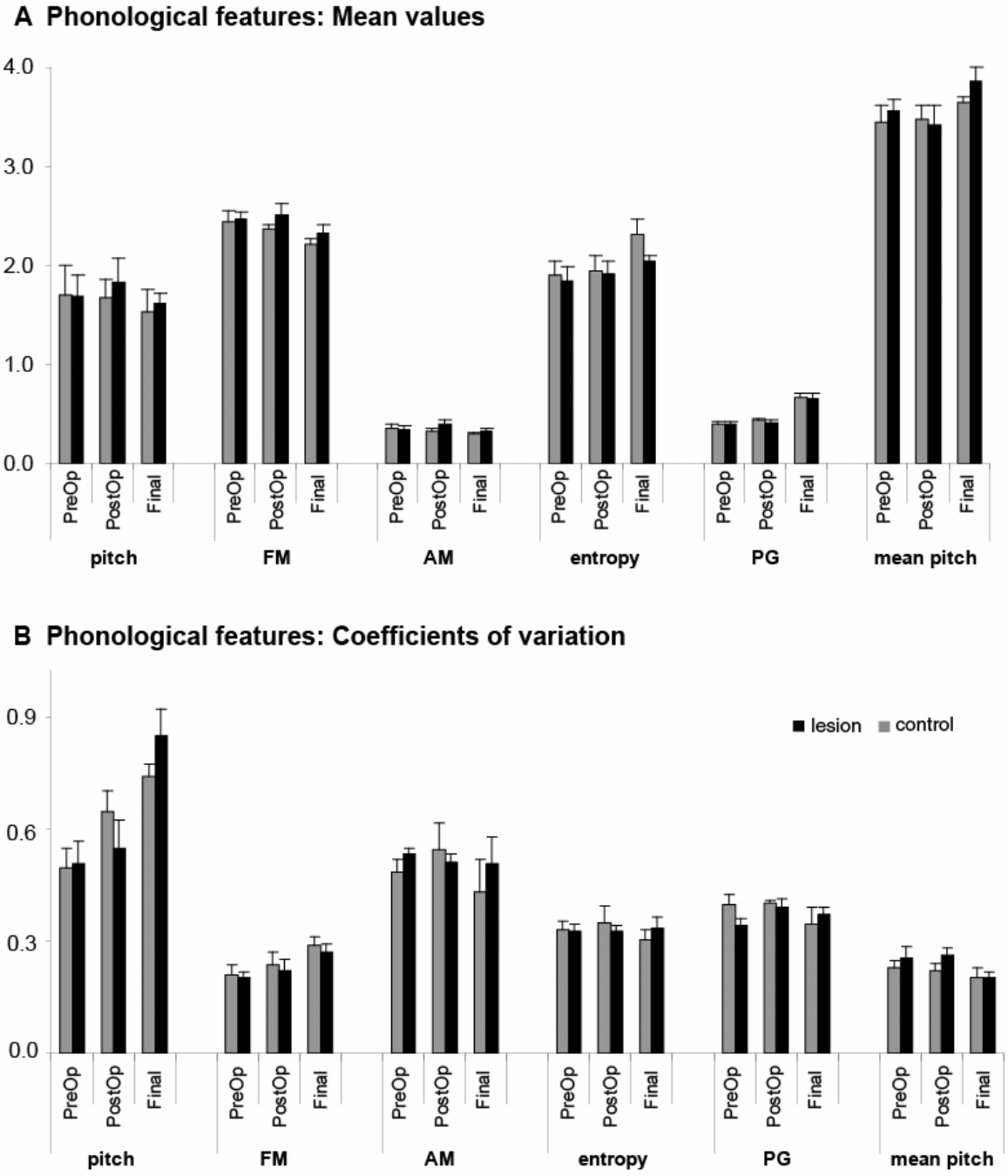
lesion song behavior of this bird at 42 days of age depicts normal subsong behavior: variable syllables are produced in long rambling strings, giving the overall song structure an amorphous quality in which there is little or no recognizable sequence[30]. This bird continued to produce normal-sounding song behavior characteristic of the transition from subsong to plastic song 8 days after the lesion, at 52 days of age. Although it is difficult to identify iterations of specific syllables at this stage of vocal development in normal birds, some of the syllables produced by this bird at 8-days post-lesion appeared similar to those produced during normal subsong prior to the lesion. However, by 15 days post-lesion (59 days of age), song behavior seemed abnormal, consisting of long strings of simple short calls interspersed with short-duration song-like syllables. By 65 days of age, the quality of this bird's song had deteriorated further (21 days post-lesion; not shown): he tended to utter strings of slightly abnormal and unstereotyped song syllables in a variable order; in addition, he continued to produce long strings of the same simple short call as previously. The song behavior of this bird did not change substantially thereafter, in the sense that somewhat atypical syllables were produced in highly variable sequences (bottom panel); the one difference was that the strings of short calls were no longer produced starting ~73 days of age. Zebra finches produce learned "long calls" outside of the song motif and this bird, like others with lesions of Ad, produced an abnormal long call that varied in duration, lacked a frequency-modulated component at onset, and was characterized by poor frequency modulation throughout (final call of bottom right panel). Y-axis is frequency (0 to 8 kHz) and X-axis is time.

We made careful qualitative assessments of the developmental recordings of each bird in order to judge the degree to which lesioned birds showed any immediate disruption in subsong behavior (see Supplementary Methods and text). Of 18 birds that were recorded within one week post-surgery (n = 6 control and n = 12 lesioned birds), no birds in either group showed any substantive changes in subsong behavior in their first PostOp recording (mean 2.0 days post-surgery). Three out of 12 birds in the lesioned group and two out of 6 birds in the control group were judged as having some minor changes from their pre-operative song behavior at latencies of 1-2 days post-surgery. However, in all five of these cases, post-lesion song behavior was not different from normal subsong behavior. Individual lesioned birds developed abnormalities at latencies ranging from 6 to 20 days post-surgery and never developed a stereotyped vocal sequence.

This lack of immediate behavioral disruption in birds with Ad lesions stands in marked contrast to that observed following lesions of LMAN in juvenile birds that are primarily centered in the core region. Previous studies of juvenile birds with LMAN lesions were made prior to the discovery of separate core and shell subregions, and encompassed all or most of the core region and varying amounts of the shell region (cf. [25, 31]). Juvenile birds with lesions targeted to $LMAN_{core}$ produce highly abnormal songs consisting of a few aberrant, highly stereotyped syllables within 24 hours post-surgery. In addition, birds with lesions aimed at $LMAN_{core}$ produce at most 2-3 different abnormal syllables. In this study, adult control birds produced a total of 6.13 $\pm$ 0.44 syllables (mean $\pm$ SEM), whereas birds in the lesion group produced 6.89 $\pm$ 0.57 syllables as adults. Thus, birds with lesion damage to Ad produced a normal number of song syllables, whereas birds with damage to the core region of LMAN produce a very small number of syllables[25, 31].

Thus, lesions of Ad in juvenile birds caused little or no immediate disruption of song, and disruption to vocal behavior increased over the course of days and weeks during the period of sensori-motor integration when normal birds are developing a stable song under the influence of motor practice and auditory feedback. The idea of the shell circuit as having an iterative evaluative function that contributes to the ability to copy vocal sounds and the refinement of vocal motor patterns from subsong ("babbling") to stable adult vocal patterns is consistent with the lack of immediate disruption to song behavior that we observed.

Supplementary Fig. S5

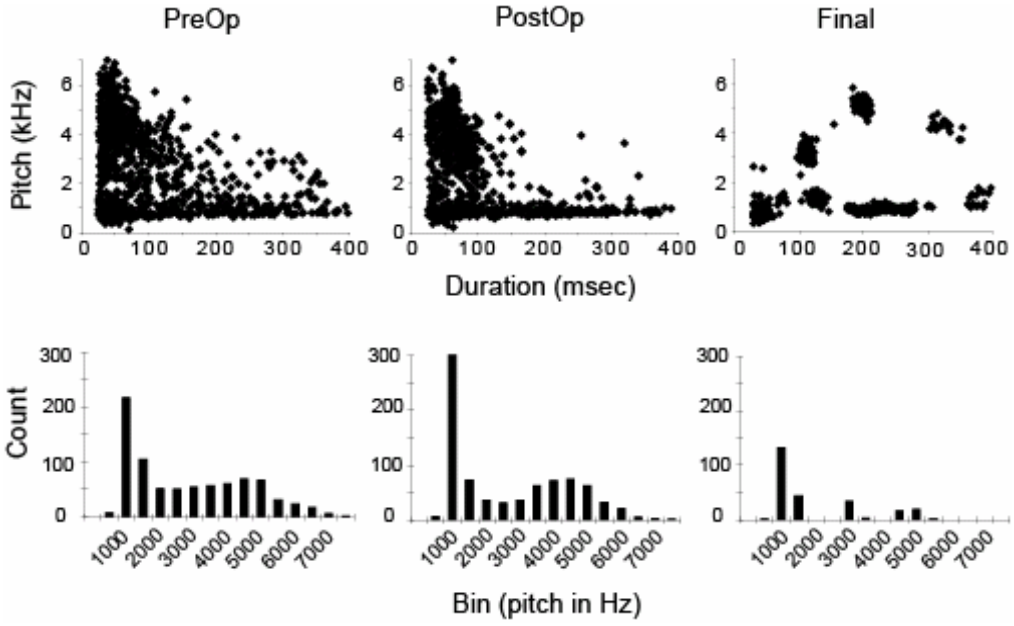


Supplementary Fig. S5. **Distribution of phonological features in lesion versus control birds**. We quantified spectral characteristics of song behavior using procedures developed by Thompson et al.[32] to parse individual song syllables and then quantify six different phonological features for each syllable in the songs of adult birds (pitch, FM, AM, entropy, pitch goodness, and mean frequency; see Supplementary Methods). (**A**) None of the six features analyzed showed substantial changes in their mean value (averaged across syllables for each bird) in either lesioned

or control birds between PreOp, PostOp, and Final recordings (although one bird in each group expanded their vocal production to include a higher range of pitch). The absence of differences in mean values for pitch at these three time points is in accord with developmental aspects of song learning showing that pitch values in juvenile birds can be highly similar to the adult pattern[30]. Values plotted are intended for relative comparison and hence are adjusted for scale (pitch, pitch goodness, and mean pitch are divided by 1,000; FM is divided by 20, and AM is multiplied by 10; see Refs. 30, 35 and http://ofer.sci.ccny.cuny.edu/sound_analysis_pro for further details concerning these measurements). (**B**) Coefficients of variation did not change for most features; the only exception was that CV of pitch increased in both groups over time. However, the CV of pitch for control birds increased between PreOp and PostOp and increased again moderately between PostOp and Final, whereas in lesioned birds pitch CV increased very little between PreOp and PostOp and then increased substantially between PostOp and Final. Wilcoxon signed-ranks tests revealed that in control birds increases from PreOp to PostOp but not from PostOp to Final were significant ($p < 0.05$), whereas in lesioned birds the increase from PostOp to Final but not from PreOp to PostOp was significant ($p < 0.05$). Adult lesioned birds (Final) tended to have a higher pitch CV than controls, and this difference was marginally significant ($U = 8.5$, $p = 0.09$). See Figure S6 for further details. Scores are depicted as mean $\pm$ SEM.

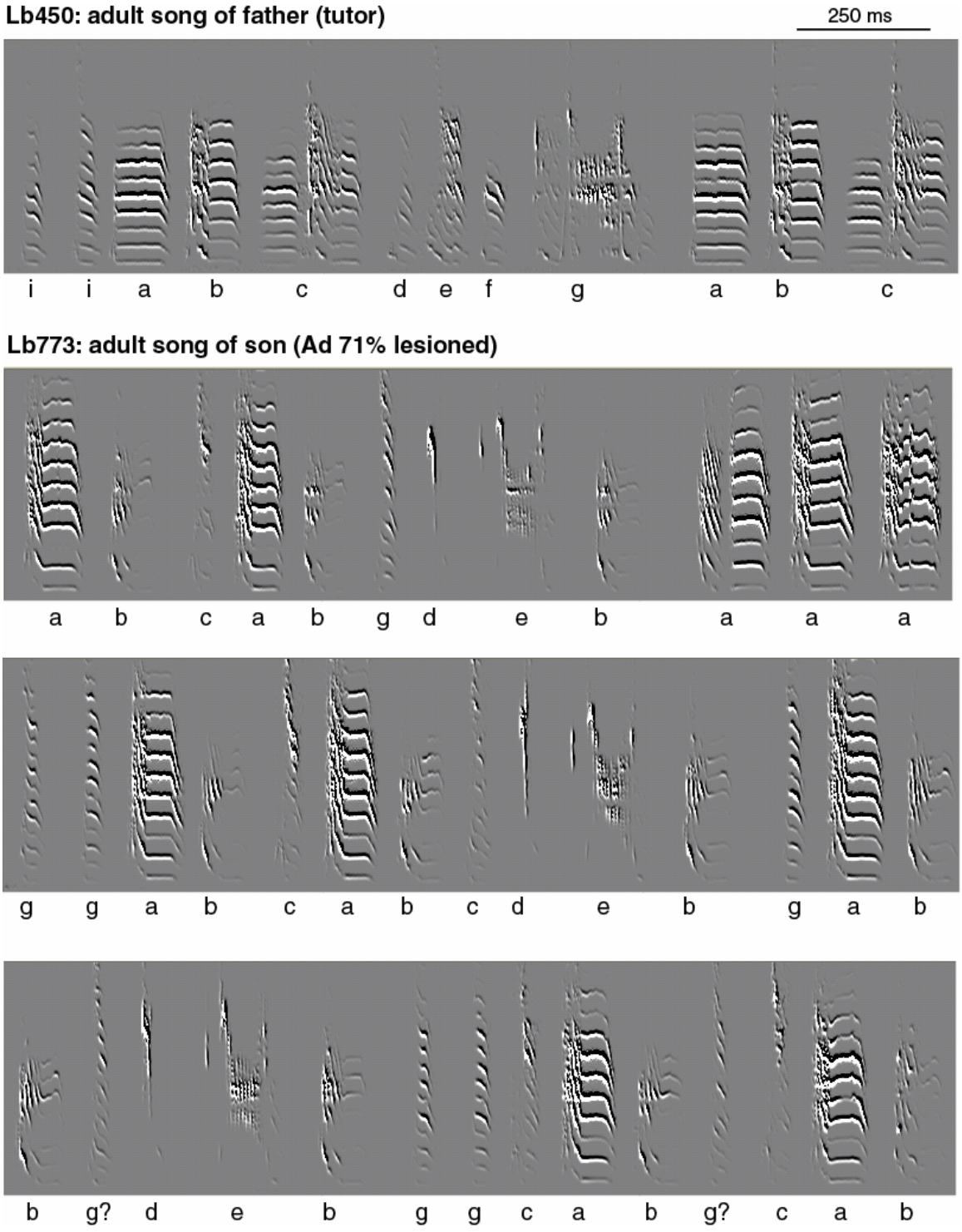
These data further demonstrate that Ad lesions do not produce disruption of song behavior within the first week post-surgery, nor do they produce gross disruption of spectral aspects of vocal motor output even in adult birds.

Supplementary Fig. S6



Supplementary Fig. S6. **Increased pitch CV in adult birds.** We examined frequency histograms of pitch values across all syllables for each bird at each recording time point as a means of evaluating the increase in pitch CV seen in both groups. These histograms revealed that the increase in CV was due to a differential distribution of pitch values at different time points. Both lesioned and control birds tended to produce a large number of syllables with a pitch of ~1.5 kHz (a range that includes introductory notes and simple song syllables) at all three time points. However, all juvenile birds (both PreOp and PostOp) showed a fairly even distribution of pitch values above 1.5 kHz, whereas adult birds showed smaller peaks at specific values corresponding to individual syllables with higher pitch. This figure shows a comparison of scatter plots for pitch (upper panels) with frequency histograms for pitch (lower panels) in a representative bird (Y782; control). The pitch CV for this bird was 0.66, 0.69, and 0.83 for PreOp, PostOp, and Final recording time points, respectively. Comparison of the two different types of plot show that the increased CV for the Final recording reflects a more heterogeneous distribution of pitch values, reflecting the specific syllables produced by this bird as an adult. Overall, these data underscore the fact that Ad-lesioned birds were capable of producing song syllables that did not differ significantly from control birds in terms of basic phonological features, and showed no tendency for immediate post-operative changes, indicating that lesions of Ad do not interfere with motor systems for song production.

Supplementary Figure S7



Supplementary Figure S7. Comparison of a tutor (father) song with that of a son with a lesion of Ad. Top panel shows a sonogram for the father (tutor) song (bird Lb450), in which syllable sequence was highly stable. Bottom panels show sonograms for one of his sons (Lb773);

this bird is the brother of Lb772 shown in Figs. S2 and S4. In this example, the syllable labeled as **a** in the songs of Lb773 is clearly similar to the one labeled **b** in the tutor song (hence it was judged as being copied). In addition, syllable **e** in the songs of Lb773 seemed similar to part of **g** in the tutor song, but was clearly not a close copy (and hence was judged as slightly similar but not similar enough to be scored as copied from the tutor; see Supplementary Methods). In several instances (such as this one), syllables produced by lesioned birds were judged as being slightly similar to tutor syllables. This is a difficult result to interpret, since it could indicate either that birds were attempting (unsuccessfully) to match to a tutor syllable, or were producing "improvised" syllables that were similar to but not a close match to tutor syllables[33]. Y-axis is frequency (0 to 8 kHz) and X-axis is time.

Our lesions were made in juvenile birds ~45 days of age, following the period of auditory learning from a tutor[34]. Therefore birds had presumably formed a neural memory of the tutor song prior to receiving a lesion of Ad. However, we do not know whether lesions of Ad impair the memory of the tutor song, or alternatively whether the template is preserved in lesioned birds but their ability to engage in auditory-motor integration is selectively disrupted. The fact that birds with Ad lesions were able to copy syllables from the tutor song in at least some instances is consistent with the idea that the template memory of the tutor song was preserved and that the function of Ad may be more related to the ability to integrate information concerning the tutor song with other types of information.

Supplementary Audio Files: example wav files of lesioned versus control birds.

These files are sample recordings of vocal behavior representative of a lack of sequence stereotypy following lesion of Ad as a juvenile (Lb772) and in a control bird (Lg676); cf. Fig. S2.

Supplementary Table S1: Summary Lesion Data and Quantitative Measures of Sequence Stereotypy in 75-day birds

control	% lesion left	% lesion right	total % lesion		S&N linearity	internal linearity	consis-tency		highest prob motif	next highest prob motif	sum of 2 highest prob motifs	basic motif	next most common motif
Lg676	0.00	0.00	0.00		1.00	1.00	1.00		0.966	0.034	1.000	abcde	xbcde
Bk867	0.00	0.00	0.00		0.86	1.00	0.93		0.548	0.387	0.935	fbcde	abcde
Y856	0.00	0.00	0.00		0.83	1.00	0.96		0.633	0.367	1.000	abcde	aabcde
R706	0.00	0.08	0.04		0.75	0.83	0.91		0.448	0.207	0.655	bcde	bycde
Y782	0.11	0.00	0.06		1.00	1.00	1.00		0.964	0.036	1.000	abcdefgh	jbcdefgh
W688	0.00	0.27	0.13		0.63	0.80	0.89		0.467	0.233	0.700	abcde	abcd
Y708	0.04	0.39	0.21		0.86	1.00	0.99		0.971	0.029	1.000	abcde	abcdef
Y717	0.40	0.10	0.25		0.75	1.00	0.98		0.867	0.067	0.934	abcd	abc
mean	**0.07**	**0.11**	**0.09**		**0.84**	**0.95**	**0.96**		**0.733**	**0.170**	**0.903**		
median	**0.00**	**0.04**	**0.05**		**0.85**	**1.00**	**0.97**		**0.750**	**0.137**	**0.968**		
SD	**0.14**	**0.15**	**0.10**		**0.13**	**0.09**	**0.04**		**0.233**	**0.150**	**0.143**		
lesion													
Lb732	0.15	0.52	0.33		0.57	0.58	0.79		0.219	0.125	0.344	(a)abcdef	abcdeff
Pu800	0.80	0.00	0.40		0.47	0.60	0.79		0.222	0.139	0.361	abcde	abcd(f)
Dg611	0.55	0.40	0.47		0.40	0.50	0.76		0.341	0.098	0.439	abcd	ecd
W671	0.31	0.63	0.47		0.50	0.60	0.79		0.212	0.091	0.303	none	a
Y753	0.42	0.55	0.48		0.44	0.50	0.80		0.171	0.143	0.314	abcdef	abccd
R757	0.54	0.64	0.59		0.65	0.67	0.90		0.477	0.091	0.568	abcdef	ghhg
Y751	0.50	0.73	0.61		0.56	0.67	0.93		0.536	0.107	0.643	abcdefg	abcdef
Lb773	0.88	0.55	0.71		0.55	0.71	0.84		0.233	0.233	0.466	abcdeb	ab
R745	0.52	0.91	0.71		0.50	0.64	0.82		0.330	0.100	0.430	abcde	abcdef
Dg674	0.77	0.87	0.82		0.40	0.47	0.76		0.435	0.174	0.609	bcde	fe
Lb740	0.88	0.90	0.89		0.42	0.50	0.85		0.448	0.103	0.551	ab?	abcdd
Lb772	0.92	0.92	0.92		0.38	0.40	0.56		0.241	0.069	0.310	abc?	abcba
Lb737	0.93	1.00	0.96		0.53	0.78	0.84		0.345	0.241	0.586	abcdef	ab
mean	**0.63**	**0.66**	**0.64**		**0.49**	**0.59**	**0.80**		**0.324**	**0.132**	**0.456**		
median	**0.55**	**0.64**	**0.61**		**0.50**	**0.60**	**0.80**		**0.330**	**0.107**	**0.439**		
SD	**0.25**	**0.27**	**0.21**		**0.08**	**0.11**	**0.09**		**0.119**	**0.054**	**0.124**		

Supplementary Table S2: Summary Lesion Data and Quantitative Measures of Sequence Stereotypy in 90-day birds

control	% lesion L	% lesion R	total % lesion		S&N linearity	internal linearity	consis-tency		highest prob motif	next highest prob motif	sum of 2 highest prob motifs	basic motif	next most common motif
Lg676	0.00	0.00	0.00		1.00	1.00	0.98		0.862	0.138	1.000	abcde	ibcde
Bk867	0.00	0.00	0.00		0.86	1.00	0.92		0.500	0.469	0.969	abcde	fbcde
Y856	0.00	0.00	0.00		0.88	1.00	0.91		0.387	0.355	0.742	abcde	afg
R706	0.00	0.08	0.04		0.78	1.00	0.93		0.516	0.387	0.903	bycde	bycdef
Y782	0.11	0.00	0.06		0.89	0.88	0.99		0.929	0.036	0.965	abcdefgh	bcdefgh
W688	0.00	0.27	0.13		0.70	0.86	0.91		0.400	0.300	0.700	abcdne	dne
Y708	0.04	0.39	0.21		1.00	1.00	1.00		1.000	0.000	1.000	abcde	n/a
Y717	0.40	0.10	0.25		0.67	0.75	0.91		0.581	0.323	0.904	abcd	abc
mean	**0.07**	**0.11**	**0.09**		**0.85**	**0.94**	**0.94**		**0.647**	**0.251**	**0.898**		
median	**0.00**	**0.04**	**0.05**		**0.87**	**1.00**	**0.93**		**0.549**	**0.312**	**0.935**		
SD	**0.14**	**0.15**	**0.10**		**0.12**	**0.10**	**0.04**		**0.246**	**0.172**	**0.116**		
lesion													
Dg611	0.55	0.40	0.47		0.45	0.67	0.74		0.281	0.250	0.531	abcde	c
W671	0.31	0.63	0.47		0.47	0.60	0.69		0.118	0.118	0.236	abbb	cadee
Y753	0.42	0.55	0.48		0.40	0.45	0.75		0.161	0.129	0.290	abcc	abc
R757	0.54	0.64	0.59		0.55	0.63	0.84		0.324	0.059	0.383	hjgabcdefx	many
Y751	0.50	0.73	0.61		0.53	0.53	0.85		0.250	0.219	0.469	abcde	abcdeg
Lb773	0.88	0.55	0.71		0.47	0.50	0.79		0.387	0.226	0.613	cdeb	gab
R745	0.52	0.91	0.71		0.38	0.44	0.73		0.182	0.061	0.243	abcde	many
Dg674	0.77	0.87	0.82		0.65	1.00	0.91		0.406	0.219	0.625	bcdefg	bcde
Lb772	0.92	0.92	0.92		0.36	0.41	0.67		0.121	0.121	0.242	ibia	ibid
mean	**0.60**	**0.69**	**0.64**		**0.47**	**0.58**	**0.77**		**0.248**	**0.156**	**0.404**		
median	**0.54**	**0.64**	**0.61**		**0.47**	**0.53**	**0.75**		**0.250**	**0.129**	**0.383**		
SD	**0.21**	**0.18**	**0.16**		**0.09**	**0.18**	**0.08**		**0.110**	**0.074**	**0.161**		

SUPPLEMENTARY INFORMATION: METHODS

Subjects and song recording

Male zebra finches were bred and raised in our group aviaries (N = 21). They were segregated from their parents and housed in individual cages starting at 38-40 days of age. Vocal behavior of each bird was recorded weekly starting at 38-42 days of age (44 kHz; Sound Analysis Pro: SAP version 1.04)[35]. All birds were recorded at least once prior to under-going surgery. Vocal behavior for all birds was recorded approximately once per week until they were at least 72-77 days of age (n = 13 lesioned birds and 8 control birds); a subset of birds were recorded until they were fully adult (approximately 90 days of age; n = 9 lesioned birds and 8 control birds). Song behavior was recorded from singly-caged birds in individual cubicles, with no females present (i.e., all songs were undirected). Birds could sometimes hear songs and calls of other birds, but could not see them. All procedures were performed in accordance with protocols approved by the USC Institutional Animal Care & Use Committee and conformed to national regulatory policies.

A minimum of 32 wav files from each weekly recording session were saved: ~8 files from the early morning and ~8 files from the early afternoon on two consecutive days. Although we did not measure rate of singing directly, we assessed the rate of singing indirectly from the 16 files we saved during each recording day by examining the time stamp of the first and last file. The time encompassed between the time stamps provides an estimate of how long it took to record the 16 files. For example, a bird that produced its first song file at 8:12 am and its 16th file at 12:36 pm required 264 minutes to produce the 16 song files that we saved on a particular day. We measured this time interval for each bird on the two consecutive days of recordings during one of the weekly sessions within the first two weeks post-surgery. We then added those times for each bird and took the median value for 5 control birds and 7 lesioned birds (in the latter case we examined birds with the largest lesions). This analysis showed that control birds required 460 minutes to record the requisite number of files whereas lesioned birds required 434 minutes to record the same number of files. Thus, lesioned and control birds clearly sang at the same rate.

Lesion procedures

Birds were anesthetized using 1.5% isoflurane and placed in a stereotaxic apparatus at ~45 days of age (mean = 45; range = 42 to 46). Ad was lesioned by ejecting 2% N-methyl-DL-aspartic acid (NMA; Sigma) in PBS through a glass micropipette (tip diameter 25-30 µM) using a Recording Nanoject (Drummond Scientific); volumes of NMA injected per unilateral Ad ranged from approximately 165 to 220 nL. Due to the difficulty of precisely targeting Ad (which is shaped like a banana), we developed the strategy of using the exact location of RA (determined by mapping the characteristic pattern of high spontaneous activity in RA neurons) to generate lesion coordinates for each bird. This strategy is predicated on the fact that Ad adjoins the lateral border of RA and arcs laterally along the dorsal arcopallium from there, and the anterior-posterior extent of RA and Ad are identical[7]. Our procedure was as follows: we lowered a pipette (attached to the Nanoject) filled with 0.2 M NaCl into RA and mapped its dorsal, ventral, and lateral borders based on recording the spontaneous activity of RA neurons. Approximately 4-5 tracks were made through RA on each side for each bird. For most birds, the recording pipette was then replaced with a pipette filled with 2% NMA and three injections of ~55 nL each were made at approximately 300-400 µM intervals lateral to the lateral border of RA (e.g., if the lateral border of RA was at 2.8 mm, then injections were made at 3.1, 3.5, and 3.9 mm lateral to the midline). The medial-most injection into Ad was made at the same depth as the center of RA, and each more lateral injection was made 300 µM more ventral. In the last six birds, the pipette was withdrawn from the brain and the NaCl solution was ejected and then refilled with 2% NMA without removing it from the Nanoject. These final birds received 6 injections of 37 nL each: the positions of lateral injections were made as in other birds, but three of these were positioned 150 µM anterior to the center of RA, and an additional three injections were positioned 150 µM posterior to the center of RA (total

volume 222 nL per unilateral Ad). Because of the duration of the procedure (i.e., including both mapping RA and lesioning Ad), each side was lesioned separately on two consecutive days (e.g., an individual bird underwent surgery on both 45 and 46 days of age). To control for possible minor damage made by the electrode tracks in RA, we mapped RA activity in an additional two birds that received no injection of NMA into Ad (but otherwise were treated identically throughout all procedures). An additional six controls were provided by those birds in which there was little or no evidence of damage to Ad (i.e., these latter birds had electrode tracks made in RA and also received NMA injections targeted to Ad but the injections either clogged or were mis-targeted). There was no difference in quantitative features of song behavior between control birds with no lesion and those with small or mis-targeted lesions, and these birds were therefore combined into a single control group (see below).

For all quantitative behavioral measures (see below), we defined birds with substantial lesion damage operationally as those with $\geq$ 30% damage to Ad, whereas control birds were those with < 30% damage to Ad. This yielded a control group in which an average of 9% Ad was lesioned (range = 0-25%; n = 8) and a lesion group in which an average of 64% Ad was lesioned (range = 33-96%; n = 13). To validate the categorization of control birds, we compared control birds with minimal lesion damage (including the two birds that did not receive NMA injections; see above) with birds that received up to 25% lesion damage: quantitative scores for control birds with the smallest amount of lesion damage (0-4%; n = 4) did not differ from birds with slightly more lesion damage (6-25%, n = 4)($p > 0.50$ in all cases; see Supplementary Table S1 for individual data).

Lesion analysis

The external borders of Ad are not well-defined in Nissl-stained sections, making it somewhat difficult to estimate the extent of the lesions. In addition, the lesions displayed some tendency to decrease in size over time, such that the visible extent of the lesion could be extremely small, even in those birds in which most of Ad was gone. In some cases in which the visible lesion extent was much smaller than the actual lesion, the arcopallium was misshapen or abnormally small overall. We verified the tendency of the lesions to disappear over time by examining the size of lesions in 4-5 adult birds within one week post-lesion; the lesion size was large in all of these birds (data not shown), indicating that gradual re-arrangements in the brain over time tended to eliminate the lesioned tissue. Because the location of Ad is strictly co-extensive with RA in terms of rostro-caudal extent (RA is clearly visible in Nissl sections) and occupies a highly consistent position within the arcopallium[7], we focused our examination on the highly characteristic area of arcopallium occupied by Ad just lateral to RA. Every section containing RA in Nissl-stained tissue was carefully examined within the area of Ad immediately lateral to RA and assigned a value between 0-100%, estimating the extent of Ad that was lesioned. Then to estimate the total percent of Ad lesioned, a weighted sum was calculated for each side: the percent Ad lesioned in each section that contained Ad was multiplied by the fraction that each section constituted of the total number of sections, and those numbers were summed to obtain the total percent Ad lesioned (the resulting percent value can range from 0 to 100). This calculation was performed separately for left and right Ad, and the percent lesioned for left and right Ad were averaged to obtain an estimate of the total lesion extent (see Supplementary Table S1). This procedure is essentially identical to procedures we used previously to assess lesion extent in MMAN[26].

As a secondary means of assessing the lesions, we attempted to label all of the LMAN axons that project to Ad[3, 4, 7]. Therefore, all birds received a second surgery between the ages of 75-100 days in which a neuroanatomical tracer was injected into LMAN; the purpose of this surgery was to try and anterogradely label any remaining (unlesioned) portions of Ad so as to help verify the extent of the lesion in each bird. LMAN was injected bilaterally with either 10% WGA-HRP in 0.05 M Tris (iontophoretically, 3 μA, 6 sec on/off for 15-20 min; Sigma) or with 10% dextran conjugated to Alexa Fluor 568 in PBS (pressure injections were made with the Nanoject using a 2

x 3 grid of 6 injections, total volume 222 nL per unilateral LMAN; Invitrogen). 48 hours following this second surgery all birds were overdosed with a barbiturate anesthetic (Equithesin), perfused, and brains were frozen-sectioned in the coronal plane at a thickness of 40 µM. Brains injected with HRP were reacted using standard techniques and alternate sections were Nissl-stained. For brains injected with dextran-Alexa, one series of tissue sections was cover-slipped with Fluoromount-G (Southern Biotech) and examined using epifluorescence microscopy and an alternate series of sections was Nissl-stained for examination under bright-field illumination. We examined the pattern of anterograde label in RA and Ad for all birds in which all or most of LMAN was covered by an injection of WGA-HRP or dextran-Alexa. Unfortunately, because the tracer injections missed part of LMAN in several birds, we could not use the pattern of label to calculate the amount of Ad remaining across all birds. However, we used the pattern of label wherever possible to verify the estimates of percent Ad lesioned in each section, and observed good agreement between the estimates made in Nissl-stained sections and the pattern of anterograde label obtained. In addition, we observed robust label within RA for any injections that included the core region of LMAN. Lastly, we carefully examined both HVC and RA to ensure that there was no damage to either of these song-control nuclei. Four birds were eliminated from consideration due to damage to HVC and surround. All analyses of lesion extent were conducted without knowledge of experimental treatment or quality of song behavior produced.

Behavioral analysis

All behavioral analyses were conducted without any knowledge of lesion extent. Overall song quality of each bird was assessed qualitatively by listening to song samples and visually examining sonograms (spectral derivatives; Sound Analysis Pro)[35] from a sample that included a minimum of 10-15 bouts of song. Song quality was carefully assessed for each bird by comparing pre- and subsequent post-operative developmental recordings. Written notes were made to indicate whether song behavior was normal or abnormal for a given stage of development, and whether vocal behavior at one recording date showed any substantive change from the previous date (e.g., post-lesion recordings made immediately following lesion of left and right sides versus pre-lesion). Final vocal recordings of birds were made between approximately 75 and 95 days of age. Although zebra finches become sexually mature ~80-90 days, we decided to perfuse some birds slightly earlier in an attempt to see whether there would be less shrinkage of the visible extent of the lesions (with limited success), and because zebra finches typically achieve a fairly stereotyped song by 70-75 days. We therefore focused on comparing the song behavior across all birds at ~75 days, using techniques described previously[25-27, 32].

Stereotypy of the temporal structure of song was quantified by making written transcripts of the sequence of syllables for at least 30 song motifs and calculating measures of linearity and consistency based on those published by Scharff & Nottebohm (1991)[25]. Briefly, measures of linearity quantify the number of different syllable-to-syllable transitions made for each bird by calculating the number of different syllables produced divided by the number of different syllable transitions (excluding introductory notes). Thus, a song motif with a fixed sequence of syllables in which the order is never varied (such as **abcde**) is perfectly linear and receives a score of 1.0. The consistency measure calculates the proportion of syllable transitions that are accounted for by the most frequent transition for each syllable (averaged across all syllables). As in previous work, we calculated two different measures of linearity: the original linearity measure devised by Scharff & Nottebohm, and an “internal linearity” measure that excludes transitions at the ends of song motifs so as to eliminate variability in the syllable that immediately preceded song terminations; this latter measure generates a perfect linearity score in cases where the sequence of syllables is invariant but the bird may truncate the motif occasionally[17, 27]. We also quantified how often specific variants of the song motif were produced (for each bird) by calculating the motif produced on the highest proportion of total motifs (highest-probability motif), as well as the motif produced with the second-highest probability[28]. These two numbers (the highest and the next-highest

probability motif variants) always account for the vast majority of motifs produced by normal birds: a normal bird with a stereotyped song produces a single (canonical) motif a high proportion of the time, but frequently also produce one motif variant. Thus the first two motif variants should account for most vocal utterances in birds with normal song behavior; these two numbers were added together to obtain the sum of the highest-probability motifs. Measurements of motif probability provide an additional measure of vocal stereotypy. We used a computer program to calculate all these measures (written by J.D. Zevin, with details, explanation and access available at http://bottjerlab.usc.edu/songinator.html). For additional details see[26, 27], and the Help notes on the Songinator website.

We quantified spectral features of song using SAP as described by Thompson et al. 2007[32]. Briefly, we parsed individual song syllables by setting syllable-threshold controls (primarily amplitude and minimum syllable and gap durations) for the adult (75-95 day old) song pattern of each individual bird such that the software reliably identified each syllable in that bird's motif. Then the Feature Batch module of SAP was used to generate measurements for six spectral features: pitch, FM, AM, entropy, pitch goodness, and mean frequency. Scatter plots using syllable duration on the X-axis and one of the six phonological features on the Y-axis were made to verify that the syllable-threshold settings accurately identified individual syllable clusters (i.e., each data point in these scatter plots represents an individual syllable, and clusters of data points correspond to repeated iterations of an individual syllable). Once we had verified that clusters within the scatter plots corresponded to different syllables in the spectrograms of each bird's song motif, we then applied these threshold settings without change for each bird to analyze songs produced prior to lesion (PreOp), within the first week post-lesion (PostOp) and during the final adult recording (Final)(approximately 32 bouts were analyzed for each recording date; in cases where birds sang less for a particular recording session we used all available bouts). PreOp recordings were made on average 2.5 days prior to the second surgery (see above) and PostOp recordings were made on average 2.0 days after the second surgery (range = 1-7 days). The resultant data set includes measurements of all six phonological features for all syllables produced at these three time points for each bird. We calculated both the mean and the coefficient of variation (CV; standard deviation divided by the mean) of each feature across all syllables (for individual birds) at each time point for control versus lesioned groups (n = 6 control and 7 lesioned birds).

We also compared the songs of experimental and control birds to those of their fathers in order to judge whether birds with Ad lesions could successfully copy a tutor model song. We identified the tutors (three different fathers) for five birds with substantial lesion damage to Ad, and made quantitative comparisons of their songs to the songs of their tutors. As a control, we also compared the songs of six birds randomly chosen from our breeding aviary that had received no experimental treatment and also compared their songs to those of their tutors (four different fathers). Our procedure was modeled after that of Basham et al. (1996)[33]: briefly, each song syllable in the son's song was compared to those in the father's (tutor) song to try and find a corresponding syllable with the highest similarity. Each syllable was given a similarity score as follows: 0 = no similarity, 1 = slightly similar, 2 = highly similar, 3 = matched. Two experimenters with no knowledge of the subjects' experimental treatment independently analyzed sonograms for each bird and these scores were averaged. Syllables with a score $\geq$2 were operationally defined as having been learned from the tutor, whereas syllables with scores <2 were judged as not having been learned from the tutor.

Mann-Whitney summed ranks tests and Wilcoxon Sign tests for related samples were used to test significance (two-tailed tests in all cases). Nonparametric tests were used because of the small sample sizes typical of this type of study and the noninterval nature of some measures.

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