

SEASONAL PLASTICITY OF SONG BEHAVIOR RELIES ON MOTOR AND SYNTACTIC VARIABILITY INDUCED BY A BASAL GANGLIA–FOREBRAIN CIRCUIT

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Abstract—The plasticity of nervous systems allows animals to quickly adapt to a changing environment. In particular, seasonal plasticity of brain structure and behavior is often critical to survival or mating in seasonal climates. Songbirds provide striking examples of seasonal changes in neural circuits and vocal behavior and have emerged as a leading model for adult brain plasticity. While seasonal plasticity and the well-characterized process of juvenile song learning may share common neural mechanisms, the extent of their similarity remains unclear. Especially, it is unknown whether the basal ganglia (BG)–forebrain loop which implements song learning in juveniles by driving vocal exploration participates in seasonal plasticity. To address this issue, we performed bilateral lesions of the output structure of the song-related BG–forebrain circuit (the magnocellular nucleus of the anterior nidopallium) in canaries during the breeding season, when song is most stereotyped, and just after resuming singing in early fall, when canaries sing their most variable songs and may produce new syllable types. Lesions drastically reduced song acoustic variability, increased song and phrase duration, and decreased syntax variability in early fall, reverting at least partially seasonal changes observed between the breeding season and early fall. On the contrary, lesions did not affect singing behavior during the breeding season. Our results therefore indicate that the BG–forebrain pathway introduces acoustic and syntactic variability in song when canaries resume singing in early fall. We propose that BG–forebrain circuits actively participate in seasonal plasticity by injecting variability in behavior during non-breeding season.

Significance Statement: The study of seasonal plasticity in temperate songbirds has provided important insights into the mechanisms of structural and functional plasticity in the central nervous system. The precise function and mechanisms of seasonal song plasticity however remain poorly understood. We show here that a basal ganglia–forebrain circuit involved in the acquisition and maintenance of

birdsong is actively inducing song variability outside the breeding season, when singing is most variable, while having little effect on the stereotyped singing during the breeding season. Our results suggest that seasonal plasticity reflects an active song-maintenance process akin to juvenile learning, and that basal ganglia–forebrain circuits can drive plasticity in a learned vocal behavior during the non-injury-induced degeneration and reconstruction of the neural circuit underlying its production. © 2017 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: birdsong, basal ganglia, seasonal plasticity, canary, variability.

INTRODUCTION

Nervous systems display a strong plasticity of structure and function that allows animals to rapidly adapt to changes in their environment. In particular, seasonal plasticity of neural circuits and behavior is often critical to survival or mating in seasonal climates (Jacobs, 1996). Temperate songbirds breed seasonally and display a pronounced seasonal plasticity in their singing behavior (Ball and Balthazart, 2010; Brenowitz and Larson, 2015). The song initially learned as a juvenile undergoes a pattern of yearly changes: birds produce highly stereotyped songs in the breeding season, then stop singing during the summer molt, resume singing in fall with short-duration songs of variable structure at lower rate and volume and gradually sing longer, louder and more stereotyped songs until the next breeding season (Nottebohm, 1981; Nottebohm et al., 1986, 1987; Guttinger, 1985; Brenowitz, 1997; Voigt and Leitner, 2008). These changes in song are accompanied by large structural reorganization of neural circuits in the brain song-control regions (Nottebohm et al., 1986; Kim et al., 1989), auditory areas (De Groof et al., 2009; Caras et al., 2012) and beyond (De Groof et al., 2008). The volume of many song-related brain regions, as well as their number of neurons and synapses, increases dramatically in anticipation of the breeding season (Nottebohm, 1981; Brenowitz, 2004), while the physiological and functional properties of their neurons are altered (Del Negro and Edeline, 2002; Del Negro et al., 2005; Meitzen et al., 2007, 2009). Songbirds have therefore emerged as a leading model for naturally occurring structural and functional plasticity in the adult central nervous system.

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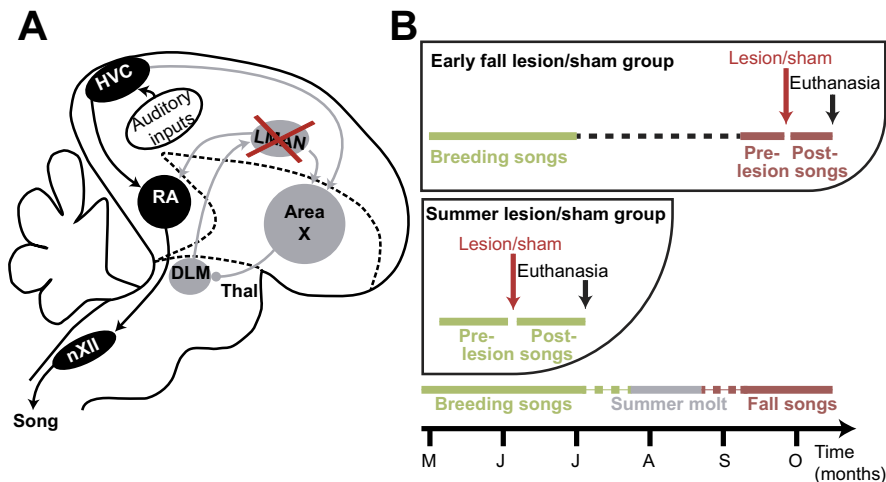


Fig. 1. Schematic view of the song system and experimental protocol. A) Schematic parasagittal representation of the song system, a network of brain nuclei centrally involved in the learning and production of song in various songbird species, including canaries. Dorsal is up, anterior is to the right. Nucleus HVC (used as a proper name) and the robust nucleus of the arcopallium (RA) constitute the motor pathway in black, while nuclei in gray form the song-related BG–forebrain loop: Area X in the BG, the medial portion of the dorsolateral nucleus of the anterior thalamus (DLM) and LMAN. In the present study, nucleus LMAN, the output of the BG–forebrain circuit, is ablated in adult canaries during the breeding season or early fall. B) Description of the timing of song recordings and lesion surgeries in the four groups of birds used in this study (breeding season LMAN- and sham-lesion; early fall LMAN- and sham-lesion). See Material and Methods for more detail on the experimental protocol.

Seasonal changes in vocal behavior, brain structure and neuronal properties are triggered at least in part by vernal increases in circulating sex steroids (Nottebohm et al., 1987; Nowicki and Ball, 1989; Fusani and Gahr, 2006; Alward et al., 2013). Interestingly, the same endocrine signals regulate juvenile song learning in seasonal as well as non-seasonal songbird species (Marler et al., 1988; Korsia and Bottjer, 1991; Whaling et al., 1995; Allienne et al., 2010). For instance, testosterone can increase song stereotypy both in seasonal plasticity (Smith et al., 1997; Tramontin et al., 2000) and juvenile learning (Heid et al., 1985; Marler et al., 1988; Gardner et al., 2005; Sizemore and Perkel, 2011). Seasonal plasticity may therefore exploit similar mechanisms as those acting during early ontogeny in juvenile learning, an idea that dates back to Ramon y Cajal (1959). However, whether seasonal song plasticity relies on the neural circuits which implement juvenile learning remains unknown.

Basal ganglia (BG) circuits promote the acquisition and maintenance of fine motor skill in vertebrates (Hikosaka et al., 2002; Grillner et al., 2005). In particular, songbirds have a specialized BG–forebrain circuit required for juvenile song learning (Fig. 1A; Bottjer et al., 1984; Scharff and Nottebohm, 1991) which generates song variability underlying vocal experimentation (Olviczky et al., 2005; Kao et al., 2005; Aronov et al., 2008). This circuit undergoes important seasonal morphological changes in seasonal songbirds (Brenowitz et al., 1998; De Groof et al., 2008), and is a target of sex-steroids (Arnold et al., 1976; Brenowitz and Arnold, 1985; Livingston and Mooney, 2001). Whether the BG–forebrain circuit is actively involved in seasonal song

remodeling, possibly injecting variability in song as in the juvenile learning process, remains however unknown. Alternatively, transient song degradation outside breeding season may rather express alteration of the motor pathway which experiences large morphological and physiological changes with seasons (Smith et al., 1997; Del Negro and Edeline, 2002; Del Negro et al., 2005; Thompson et al., 2007; Meitzen et al., 2007, 2009), and is an important target of brain sex steroids (Ball and Balthazart, 2010; Brenowitz, 2015).

Here, we sought to determine whether seasonal song plasticity relies on the song-related BG–forebrain circuit. We compared the temporal, acoustic and sequencing properties of canary song between the breeding season, when song is most stereotyped, and just after resuming singing in early fall, when canaries sing their most variable songs and may add new syllables to their repertoire. We then assessed how lesions of the output of the song-related BG–forebrain circuit,

the magnocellular nucleus of the anterior nidopallium (LMAN), interfere with seasonal changes. While having no effect on song during the breeding season, LMAN lesions drastically reduced song acoustic variability, increased song and phrase duration, and decreased syntax variability in early fall, reverting at least partially seasonal changes observed between the breeding season and early fall.

EXPERIMENTAL PROCEDURES

Animals

Fourteen adult domesticated male canaries (*Serinus canaria*), 1–3 years of age, were used for this experiment. All canaries were reared in the laboratory and belonged to an outbreed form of the common canary with a heterogeneous genetic background. Birds had constant access to seeds (mainly canary grass, *Phalaris canariensis*, and rape, *Brassica rapa*), crushed oyster shell and water. Seeds supplemented with fresh food and water were provided daily. The birds were housed on a natural photoperiod (both in the aviary and in sound isolation boxes during the experiment), with an air temperature maintained at 21 ± 2 °C. Animal care and experiments were carried out in accordance with the European directives (86/609/CEE and 2010-63-UE) and the French legislation. Experiments were approved by Paris Descartes University (project authorization n° 13–092) and Paris Centre et Sud ethics committees (project authorization n° 2012–0069).

Song recordings and protocol timeline

In May (the breeding season), we selected 14 males from our aviary for song recordings. Birds were individually housed in sound isolation chambers (custom built) and their spontaneous vocalizations were continuously recorded using RØDE M3 microphones, an external sound card for microphone amplification and power (M-Audio Fast Track Ultra 8R), and the software Sound Analysis Pro 2011 (SAP, Tchernichovski et al., 2001). SAP parameters were set with conservative thresholds (software threshold to 4–6) in order to record the initiation of canary's vocalization which can be very low in volume, especially in early fall when song is lower in volume and harder to detect than during the breeding season song.

After being held for a period of at least 2 weeks to collect songs, a subset of four birds underwent a lesion/sham surgery targeted at the LMAN (3 with LMAN lesion, 1 with sham-lesion). After recovery from the lesion-related surgery, the birds were held in the recording chambers for at least 2 weeks to collect song recordings. Additionally, two control birds were recorded over the same time period. At the end of the breeding season (late June or early July), all 6 birds were deeply anesthetized and euthanized to extract the brain.

The other birds ($n = 10$) that were recorded during the breeding season were transferred to the aviary for the summer, where their singing behavior was regularly monitored, confirming that they did not resume singing. At the end of August, before any of the previously recorded birds had resumed singing, birds returned to individual sound isolation chambers for continuous recording of their spontaneous vocalizations, also first confirming that they had not resumed singing yet. Seven out of ten birds received a LMAN lesion ($n = 4$) or sham lesion surgery ($n = 3$) five days after the detection of their first song. Birds were then placed back in the recording chambers again for another 1–3 weeks of song recordings before undergoing a dye injection surgery and being euthanized and their brain extracted for histological investigation (see below). We refer to these birds as “Early fall” birds to indicate that we focused on the first few days/weeks of singing following the long silent period during summer. A timeline for the experiment is recapitulated in Fig. 1B.

During the continuous recordings, isolation chambers were open periodically so the birds could hear one another (1–2 h, 2–5 times a week). These regular periods of social interactions prevented the negative effects of social isolation. After the end of post-lesion recordings, the birds underwent a dye injection surgery and/or were euthanized and their brain extracted for histological investigation (see below).

Song analysis

Songs were sorted from continuous recordings based on sound amplitude and spectral content using a custom Matlab script. All vocalizations that could be distinguished from calls (many call types have been described in canaries: Mulligan and Olsen (1969), Nagle

et al. (2002)) and that were composed of at least two continuous and different types of acoustic elements, called syllable types, were considered as being songs. As a consequence, bouts of vocalizations where the total duration was greater than 2 s were included in our dataset, regardless of the season. Note that the song of domesticated canaries has a characteristic acoustic structure and temporal pattern that have been described in numerous studies (Del Negro et al., 2005; Lehongre et al., 2008; Markowitz et al., 2013). It takes the form of a complex sequence, made up of multiple distinct phrases, where each phrase type is often composed of the repetition of a syllable type generally formed of 1 or 2 notes (see two examples of songs produced by a given bird in Fig. 2). During the breeding season, male canaries sing loudly and their songs show a high degree of stereotypy (highly stereotyped songs). Repetitions of a given syllable type within a phrase are highly similar to one another (Fig. 2A). One other characteristic of the song of domesticated canaries is the diversity of syllable types (or phrase types, given that a phrase consists of a repetition of a single syllable type) that form songs. During the breeding season, the adult repertoire usually contains between 20–30 distinct syllable/phrase types.

To determine the effect of the LMAN lesion on song features, we analyzed songs produced during at least 3 distinct days of the 10-day period preceding the lesion surgery and during at least 3 distinct days of the 10-day period following the first day of singing after lesion surgery (days 1 of these two time periods were always selected; the day selection depended on whether the bird sang or not). About 30–50 songs per day were used in this analysis.

To analyze canary song features, we used a custom script in Praat software (Boersma and Weenink, 2010) that first opened recorded files that contained at least one song to create spectrograms. Both the beginning and the end of each song phrase were marked by visual inspection of the sonogram and the corresponding oscillogram. Within a given canary song phrase, the first syllable and the last one are generally slightly different from the other repeated syllables, these variations probably allow transition between phrases. However, as similar variations in syllable features can be observed each time a given phrase is produced, each phrase can be assigned a label corresponding to the phrase type and a repertoire of phrase types can be created. Yet, these variations in syllable features within a given phrase type make it quite difficult to perform automatic clustering. Syllable sorting can be only partially automated through cluster analysis in the breeding season song (results not shown, but see Markowitz et al., 2013 for semi-automated clustering of canary song syllables in the breeding season). In early fall song, the boundaries between syllable types fluctuate a lot, and the low amplitude of syllables whose spectral features are often distorted make it virtually impossible to sort syllable types automatically. The repertoire of the different syllable types (or phrase types, given that a phrase consists of a repetition of a single syllable type) per individual bird and per season was thereby established.

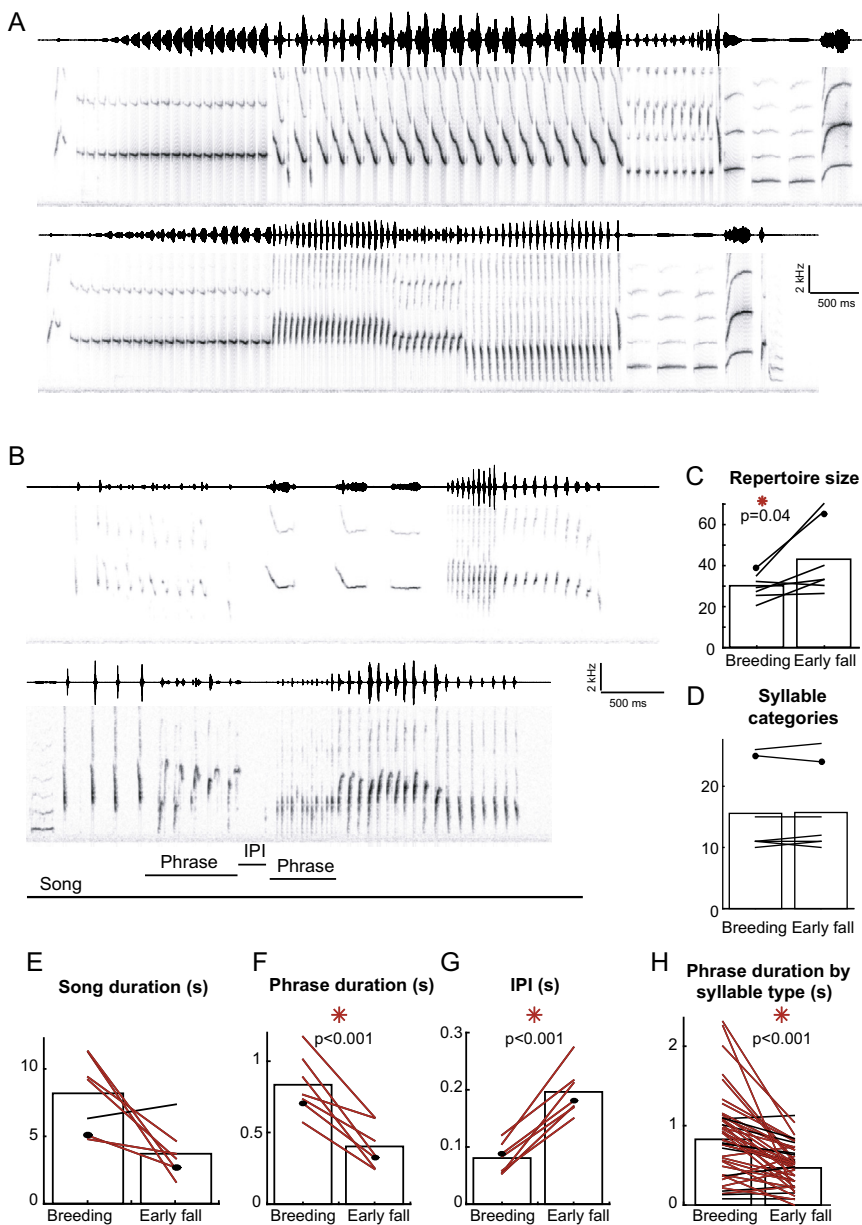


Fig. 2. Songs are shorter, with shorter phrases and longer inter-phrases intervals (IPI) in early fall. A) Oscillograms and spectrograms of two example songs produced in the breeding season. B) Oscillograms and spectrograms of two example songs produced in early fall, in the first days following resuming singing after the summer. Horizontal lines below the example song spectrogram illustrate the terminology used throughout the paper: a song can be decomposed into phrases, which consist in multiple repetitions of a single syllable type, and are separated by IPI. In C–F, black disks depict the data from the example bird B9 (also used as an example in Figs. 3 and 5). C) The repertoire size slightly increases from the breeding season to early fall ($n = 7$). D) Total number of syllable categories in each bird recorded in the breeding season and early fall ($n = 7$). Syllable categories, which group several similar syllable types, do not change in number from the breeding season to early fall. C and D) Black disks indicate the corresponding data of the example bird from Fig. 1 (from which an example transition is shown in A). E) Song duration is reduced in early fall compared to the breeding season. F) Phrase duration is strongly reduced in early fall compared to the breeding season, when compared for each bird separately. G) IPI duration is strongly increased in early fall compared to the breeding season. H) Phrase duration is strongly reduced in early fall compared to the breeding season, when compared for all syllable types in all birds. E–H: Red and black lines denote a within-subject (or within syllable type for E) significant change ($p < 0.05$) or no significant changes ($p > 0.05$), respectively, in the considered song feature.

The visual classification of syllables into syllable types constituting the repertoire was based on spectral and temporal parameters and repetition rate. The custom-written Praat script allowed creating multiple files that included a sound file per song and per song phrase. Song and phrase lengths, times of phrase initiation and end, as well as phrase sequences were stored in a data file for further analysis.

To precisely quantify variability in syllable features within phrase types, another custom-written Praat script opened sound files that contained individual song phrases to perform continuous pitch measurements on syllables and thereby to extract pitch traces (frequency range 200–10 000 Hz; analysis method: auto-correlation; Octave cost 0.1; Octave-jump cost 0.1; Voiced/unvoiced cost 0.1). Data were exported into text files and subsequently imported into MATLAB. When the unit syllable was repeated at least ten times within the song phrase, all pitch traces of the syllable type were aligned using the following procedure. We first calculated the average of all pitch traces that were previously cropped to the length of the shortest iteration. The average pitch trace was prolonged to the length of the longest pitch trace by a polynomial extrapolation. All traces were individually aligned by calculating the jitter that minimized the distance to the average extrapolated trace. The point-by-point average, standard deviation (SD) and the coefficient of variation (CV) of all aligned pitch traces were calculated over all iterations of a given syllable type in a given condition. The mean of the average pitch trace over all time points is referred to in the following as “mean of pitch traces”, while the average of the CV of aligned pitch traces over all time points is referred to as “CV of pitch traces”. Additionally, for syllable displaying a stable ending at constant pitch, the mean of average aligned pitch traces over the last five time points was referred to as “mean of ending pitch”, while the average of the CV

of aligned pitch over the last five time points was referred to as “CV of ending pitch”.

The song of the domesticated canary consists of phrases that are arranged in flexible sequences (Del Negro et al., 2005; Lehongre et al., 2008; Markowitz et al., 2013). To quantify variability in ordering of phrases within songs, the entropy of a convergent (resp. divergent) transition point to (resp. from) syllable i in the song sequence is calculated as the sum of $-p_j \log(p_j)$ for all possible preceding (resp. following) syllable types j , where p_j denotes the probability to have syllable type j preceding (resp. following) syllable type i .

We also conducted a Principal Component Analysis (PCA) on all song parameters quantified in the three different conditions (breeding condition, early fall before lesion and after lesion) for early fall LMAN- and sham-lesion birds: repertoire size, song duration, phrase duration, IPI duration, mean pitch, CV of pitch, entropy of convergent transitions, entropy of divergent transitions. For each bird and each song parameter, the mean values from the breeding season and early fall after lesion were divided by the mean value in early fall before lesion. This “pre-lesion normalization” was intended to ease visualization and reveal whether LMAN lesion shifted song parameters closer to breeding season values.

Surgical procedures

Electrolytic lesions. Each animal was first food deprived for 30 min. An analgesic was administered before starting the surgery (meloxicam, 5 mg/kg). The anesthesia was then induced with a mixture of oxygen and 3–5% isoflurane, until the birds lost their muscular tonus. Birds were then moved to the stereotaxic apparatus with the beak at an angle of 45° downward from the horizontal, where they were kept under anesthesia with a mixture of oxygen and 1–1.5% isoflurane. Local anesthetic (1% lidocaine) was injected under the scalp and small craniotomies were made above the midline reference point, the bifurcation of the midsagittal sinus, and above the lateral magnocellular nucleus of the anterior nidopallium (LMAN) bilaterally. A tungsten microelectrode (FHC) was then lowered to six different locations on each side of the brain to cover the full area of LMAN. In a pilot study, we started with the procedure described by Burt et al. (2000) to lesion LMAN in female canaries. The authors delivered 400 μ A of current for 1 min at four stereotaxic coordinates. Given the histological results of this pilot study, coordinates were updated, the number of sites where the current was delivered through the tip (from 4 to 6) was increased, and the total amount of current delivered to each site to lesion LMAN was reduced. At each of the six sites, we delivered 100 μ A of current through the tip. For sham lesion, the same procedure was applied, except that no current was applied through the electrode. The coordinates chosen for the sites of electrolytic lesions were the following ([Antero-posterior; Lateral; Deep] compared to midline reference point): [4.5; 1.7; three depths: 2.1, 2.3 and 2.5] – [4.5; 1.2; 2.3]

– [4.5; 2; 2.3] – [5; 1.7; 2.3]. To perform sham lesions, the same procedure was used, except that no current was delivered through the electrode.

Dye injection. To confirm that LMAN lesion induced loss of LMAN-to-RA projecting neurons, three birds received fluorescent tracer injections in the robust nucleus of the arcopallium (RA) before the end of the experiment. We performed iontophoretic injections of fluorescent dye (5% Alexa-488- or -568-conjugated 10 kDa dextran amine in 0.01 M phosphate-buffer PB, pH 7.4, injected by 10 μ A DC pulses of 10-s duration, 50% duty cycle for 2 min) through a glass pipette with a small (> 10 μ m) tip, using the following coordinates: 1.1 mm posterior, 2.5 mm lateral and 2 mm deep. After a survival time of 3–5 days to allow for axonal transport of the dyes, birds were euthanized as described below.

Histology. Animals were euthanized by intramuscular injection of sodium pentobarbital (Nembutal) and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde as fixative. The brain was then removed, post-fixed in 4% paraformaldehyde for 24 h, and cryoprotected in 30% sucrose. 60- μ m-thick sections were then cut in the parasagittal plane on a freezing microtome and processed for histological examination to verify the location of the lesion and fluorescent dye. Alternating series of brain slice underwent Nissl-stain treatment or were simply mounted for to visualize the fluorescent tracer using a fluorescence microscope.

The lesion quantification was assessed by visual inspection of the Nissl-stained slices. For each brain hemisphere, the overall extent of LMAN lesion was estimated from the number of slices where LMAN was intact, partially ablated or totally absent (0 if LMAN was not affected by the lesion, 50% if LMAN was still clearly visible in some slices but missing and/or overlapping with the lesion in others, and 100% if LMAN was totally ablated). This resulted in a 0–100% range measure for the lesion size across all birds.

Data analysis

Statistics. Numerical values are given as mean \pm SD, unless stated otherwise. Song, phrase and interval durations, repertoire size, mean syllable frequency and the coefficient of variation (CV) of their frequency were compared between various conditions: 1/before and after lesion for birds which received a lesion during the breeding season and their corresponding controls, 2/in the breeding season, early fall before lesion and early fall after lesion for birds which received a lesion during fall and their corresponding controls. All statistical tests were applied using Matlab predefined functions (Matlab 2014b, Mathworks).

For lesions performed during the breeding season, there are only two conditions to compare and the null hypothesis was that LMAN lesion does not change song properties. To test this hypothesis, the data collected from each bird were compared before and after lesion with a Wilcoxon signed rank test (for song duration,

Table A. Data from single birds from the LMAN- and sham-lesion groups in early fall. The temporal parameters of song (song duration, phrase duration, song length in number of phrases and IPI duration) are averaged within each subject and condition, to reveal significant effects of lesion and season. For each case, a 3-way ANOVA is run to determine whether a significant change can be found between any conditions, followed by post hoc multiple comparison Tukey's tests to reveal specific effects of season and/or lesion

Parameter	Bird	Breeding		Fall Pre		Fall post		Stat tests	F	p-value	Post-hoc test	Post-hoc p values				
		Sample size	Value	Sample size	Value	Sample size	Value					Season effect (Br vs Fpre)	Season and lesion effects (Br vs Fpost)	Lesion effect (Fpre vs Fpost)		
Song duration	Sham 1	30	11 ± 4 s	67	1.6 ± 1 s	112	1.4 ± 0.8 s	3-way ANOVA	379	9×10^{-70}	Tukey's test	p < 10⁻⁶	p < 10⁻⁶	0.8		
	Sham 2	67	6 ± 4 s	118	5 ± 5 s	100	10 ± 5 s					19.1	2×10^{-8}	0.3	p < 10⁻⁶	p < 10⁻⁶
	Sham 3	34	9 ± 5 s	81	5 ± 3 s	61	4 ± 3 s					31.4	2×10^{-12}	p < 10⁻⁶	p < 10⁻⁶	0.4
	Lesion 1	182	5 ± 4 s	181	3 ± 1 s	392	5 ± 4 s					40.6	2×10^{-17}	p < 10⁻⁶	0.5	p < 10⁻⁶
	Lesion 2	29	11 ± 7 s	63	3 ± 2 s	15	9 ± 4 s					39.4	2×10^{-13}	p < 10⁻⁶	0.3	p < 10⁻⁶
	Lesion 3	50	9 ± 7 s	70	3 ± 2 s	30	6 ± 3 s					21.1	9×10^{-9}	p < 10⁻⁶	0.06	0.007
	Lesion 4	58	5 ± 2 s	70	4 ± 2 s	73	5 ± 2 s					7.33	8×10^{-4}	0.01	0.9	0.001
	Sham 1	312	1 ± 0.5 s	215	0.3 ± 0.2 s	354	0.4 ± 0.2 s					367	1×10^{-116}	Tukey's test	p < 10⁻⁶	p < 10⁻⁶
Phrase duration	Sham 2	512	0.7 ± 0.4 s	212	0.6 ± 0.3 s	271	0.6 ± 0.4 s	34.8	2×10^{-15}	Tukey's test	p < 10⁻⁶	p < 10⁻⁶	0.3			
	Sham 3	398	0.7 ± 0.4 s	205	0.4 ± 0.4 s	277	0.5 ± 0.5 s	41.7	5×10^{-18}	p < 10⁻⁶	p < 10⁻⁶	0.2				
	Lesion 1	1310	0.7 ± 0.6 s	1214	0.3 ± 0.3 s	4032	0.5 ± 0.4 s	287	4×10^{-120}	p < 10⁻⁶	p < 10⁻⁶	p < 10⁻⁶				
	Lesion 2	327	0.9 ± 0.5 s	333	0.3 ± 0.2 s	234	0.5 ± 0.3 s	291	7×10^{-98}	p < 10⁻⁶	p < 10⁻⁶	p < 10⁻⁶				
	Lesion 3	381	1.2 ± 0.8 s	311	0.6 ± 0.4 s	209	0.8 ± 0.5 s	72.6	6×10^{-30}	p < 10⁻⁶	p < 10⁻⁶	0.0009				
	Lesion 4	444	0.6 ± 0.4 s	596	0.2 ± 0.2 s	672	0.4 ± 0.3 s	159	5×10^{-64}	p < 10⁻⁶	p < 10⁻⁶	p < 10⁻⁶				
	Sham 1	30	11 ± 4 phrases	67	4 ± 1 phrases	112	4 ± 1 phrases	149	7×10^{-41}	Tukey's test	p < 10⁻⁶	p < 10⁻⁶	1			
	Sham 2	67	9 ± 4 phrases	118	13 ± 9 phrases	100	12 ± 8 phrases	6.82	0.001	0.001	0.06	0.9				
Song length (nb phrases)	Sham 3	34	12 ± 7 phrases	81	10 ± 6 phrases	61	8 ± 5 phrases	1.75	0.2	0.4	0.2	0.9				
	Lesion 1	182	8 ± 4 phrases	181	7 ± 3 phrases	392	10 ± 5 phrases	45.3	3×10^{-19}	0.3	p < 10⁻⁶	p < 10⁻⁶				
	Lesion 2	29	12 ± 7 phrases	63	6 ± 4 phrases	15	16 ± 9 phrases	20.5	3×10^{-8}	0.0001	0.1	p < 10⁻⁶				
	Lesion 3	50	7 ± 5 phrases	70	5 ± 3 phrases	30	8 ± 4 phrases	6.28	0.002	0.005	1	0.02				
	Lesion 4	58	8 ± 3 phrases	70	9 ± 4 phrases	73	10 ± 4 phrases	4.04	0.02	0.1	0.01	0.6				
	Sham 1	282	0.05 ± 0.07 s	148	0.2 ± 0.4 s	242	0.1 ± 0.2 s	22	6×10^{-10}	Tukey's test	p < 10⁻⁶	0.0004	0.05			
	Sham 2	445	0.05 ± 0.1 s	201	0.2 ± 0.6 s	247	0.1 ± 0.1 s	12.8	3×10^{-6}	p < 10⁻⁶	0.007	0.2				
	Sham 3	364	0.06 ± 0.09 s	184	0.2 ± 0.1 s	248	0.2 ± 0.2 s	62.5	6×10^{-26}	p < 10⁻⁶	p < 10⁻⁶	0.06				
IPI duration	Lesion 1	1128	0.09 ± 1 s	1034	0.2 ± 0.2 s	3640	0.1 ± 0.1 s	144	7×10^{-62}	p < 10⁻⁶	0.02	p < 10⁻⁶				
	Lesion 2	298	0.1 ± 0.1 s	271	0.3 ± 0.7 s	221	0.1 ± 0.2 s	13.9	1×10^{-6}	p < 10⁻⁶	1	0.0001				
	Lesion 3	332	0.08 ± 0.08 s	243	0.2 ± 0.4 s	179	0.1 ± 0.2 s	9.43	9×10^{-5}	0.0001	0.05	0.3				
	Lesion 4	386	0.1 ± 0.2 s	526	0.2 ± 0.2 s	599	0.2 ± 0.2 s	28.7	6×10^{-13}	p < 10⁻⁶	p < 10⁻⁶	0.2				

phrase duration and number per song, interval duration). As the effect of lesion was consistent among various individuals, we also pooled results from all syllable types and applied Student t-test to compare the two conditions among all song phrases after checking that samples were normally distributed and with similar variance (for phrase duration, pitch CV and mean, transition entropy).

For birds of the early fall lesion/sham groups, that were recorded both during the breeding season and early fall, we ended up with three conditions (breeding, early fall before lesion/sham, early fall after lesion/sham). Two null hypotheses were tested on these data sets: (i) that there is no difference in song between seasons, and (ii) that LMAN lesion does not affect song in early fall. We first applied three-way analysis of variance (ANOVA) on the data sets to check for differences between all three conditions. Again, the statistics were either run among data collected in single birds (for song duration, phrase duration and number per song, interval duration), or, as the various syllable types in different birds did not display different results, we pooled all syllable types and tested for differences between all conditions among all syllable types (for phrase duration, pitch CV, pitch mean and mean transition entropy). Finally, we performed post hoc pair comparisons (Tukey's test for multiple comparison) between the breeding season and early fall (before lesion/sham) and between before and after lesion/sham in early fall.

For the sake of clarity, the results of statistical tests for individual birds are only briefly mentioned in the text (Results), but all results and statistical tests are reported in a separate table (Table A in Appendix), with the number of measurements (n), the type of test used, the associated p -value (the probability of observing the given result, or one more extreme, by chance if the null hypothesis is true), and degrees of freedom for t -tests (df).

RESULTS

Song structure and repertoire

The canary has a fairly complex song structure (Güttinger, 1979; Del Negro et al., 2005; Markowitz et al., 2013). Canary song consists of a series of phrases, where each phrase is composed of the repetition of a syllable generally formed of 1 or 2 notes (Fig. 2A). A male canary has a repertoire of around 20–30 distinct phrases (range 12–40 in the breeding season). Only a part of the repertoire (on average, 8–10 phrases) is present in each song, and phrases may be recombined in different ways. There is thus no fixed repertoire of song types. The succession of phrases in a song is constrained by some form of syntactic organization, with sequences of a given number of phrases (varying from 2 to 6) that regularly occur in songs of the same individual (Del Negro et al., 2005; Lehongre et al., 2008; Markowitz et al., 2013). In the present study, syllables were classified into syllable types based on several features: the number of notes that characterize the syllable, the shape, the frequency bandwidth (minimal and maximal frequency), the syllable duration

and the repetition rate, as described in the literature. The same criteria were used to assess the impact of LMAN lesions on songs produced either in the breeding season song (Fig. 2A) or in early fall song (Fig. 2B).

While some syllable types were common between the two seasons, others were produced only in one condition. In addition, due to an increase in the acoustic variability of syllables (as described below), the bird's repertoire increased in early fall (from 29.1 ± 6.6 to 42.4 ± 18.2 syllable types, $n = 7$ birds, Wilcoxon signed-rank test, $z = 2.2$, $p \leq 0.05$, $df = 6$, Fig. 2C). We also determined larger syllable categories based only on the shape of pitch contours (falling in four groups: increasing pitch, decreasing pitch, flat or non-monotonic) and repetition rate. These large categories thereby included multiple syllable types that differed in variable syllable duration and frequency range. The number of these larger syllable categories remained stable from the breeding season to early fall (from 15.6 ± 7.0 to 15.7 ± 6.9 large syllable categories, $n = 7$ birds, Wilcoxon signed-rank test, $z = 0.7$, $p > 0.05$, $df = 6$, Fig. 2D).

A subset of syllables that were sung in early fall were considered as being special and were not classified as being a syllable type because they did not resemble any typical canary syllable types. They account for only a very small percentage of vocalizations (on average $2.1 \pm 1.9\%$ of recorded vocalizations; range: 0–4%), regardless the group of birds (lesion and sham-lesion birds) and the period (before or after the lesion).

While previous reports have highlighted the overall seasonal changes in the song of canaries (Güttinger, 1985; Nottebohm et al., 1986, 1987; Del Negro et al., 2005; Voigt and Leitner, 2008), we performed a systematic quantification of the changes undergone by canary song between the breeding season and the short period of time immediately following the long summer pause that we called early fall (see Material and Methods). We followed three main axes of analyses. First, we looked at song structure, measuring song and phrase duration, as well as the duration of silent intervals between phrases (inter-phrase interval, IPI). Secondly, we precisely characterized syllables on the basis of their acoustic parameters: fundamental frequency of each syllable rendition and its time course (called hereafter "pitch trace", see Methods). We paid attention to the variability of these characteristics from rendition to rendition. Thirdly, we focused on phrase ordering within songs taking into account each syllable/phrase type that appeared more than five times in our data set. We quantified the number of distinct syllable types preceding a given syllable type and their respective probabilities (convergent transition; if the syllable type "a" is always preceded by the syllable type "b", the convergent transition of the syllable type "a" is 1.0) and the number of distinct syllable types following a given syllable type and their respective probabilities (divergent transition; if the syllable type "a" is always followed by the syllable type "b", the divergent transition of the syllable type "a" is 1.0) for all syllable types. Transitions with probabilities lower than 2% were excluded to avoid contamination by unlikely category transitions.

Importantly, an accurate estimation of both acoustic syllable variability within phrases and sequence transition probabilities in canary song required the analysis of a large data set to include many renditions of each phrase type. The number of song phrases produced by a given bird in a given condition that were identified and analyzed ranged from several hundred to few thousand of phrases (breeding season, 13 birds, mean \pm SD per bird: 628 ± 419 , range 383–1556; early fall lesion, 7 birds, 553 ± 410 , range 233–1409; early fall post-lesion/post-sham, 7 birds, 1013 ± 1546 , range 456–2865).

Songs are shorter and more variable in early fall compared to the breeding season

Fig. 2 displays two representative examples of songs produced by one individual, one recorded during the breeding season (Fig. 2A) and one recorded during early fall (Fig. 2B). These examples illustrate the most drastic changes occurring between the breeding season and early fall, namely the decreased overall song duration, the shortening of individual song phrases and the lengthening of intervals between phrases, and, most strikingly, the increased variability between various renditions of the same syllable within a given phrase.

As previously described (Del Negro et al., 2005; Voigt and Leitner, 2008), we found that song duration was significantly shorter in early fall compared to the breeding season over all birds considered in this study ($n = 7$ birds, breeding season: 8.0 ± 2.7 s, early fall: 3.6 ± 1.8 s, Wilcoxon signed-rank test, $z = 2.7$, $p \leq 0.01$, $df = 6$; Fig. 2E). Importantly, within subject, there was a significant decrease in song duration in six out of the seven birds (see Table A in Appendix for results and statistics in individual birds). This shortening of songs was due to a reduction in the duration of single song phrases rather than to a decrease in the number of different phrase in each song. Indeed, on the one hand, the duration of single song phrases was significantly decreased in early fall as compared to the breeding season in all seven birds considered ($n = 7$ birds, breeding season: 0.8 ± 0.2 s, early fall: 0.4 ± 0.2 s, Wilcoxon signed-rank test, $z = 2.8$, $p \leq 0.01$, $df = 6$; see Table A in Appendix for results and statistics in individual birds, Fig. 2F; among all $n = 45$ syllable types produced in both conditions: 0.8 ± 0.5 s vs 0.5 ± 0.3 s, paired t-test, $t = 5.3$, $p \leq 0.001$, $df = 44$, Fig. 2H). On the other hand, there was no consistent change in the number of phrases per song between the breeding season and early fall ($n = 7$ birds, breeding season: 9.8 ± 2.2 ; early fall: 8 ± 3.1 phrases per song, Wilcoxon signed-rank test, $z = 1.2$, $p > 0.05$, $df = 6$; see Table A in Appendix for results and statistics in individual birds). Unlike song phrases, the silent intervals between phrases (IPI) were lengthened in early fall as compared to the breeding season in all 7 birds (among $n = 7$ birds, breeding season: 0.08 ± 0.03 s, early fall: 0.2 ± 0.04 s, Wilcoxon signed-rank test, $z = 3.1$, $p \leq 0.01$, $df = 6$; see Table A in Appendix for results and statistics in individual birds, Fig. 2G).

More importantly, our results show for the first time that the acoustic variability of syllables across repetitions within song phrases was dramatically increased in early fall compared to the breeding season in canaries. This increase in acoustic variability, illustrated on Fig. 3, was quantified by extracting the continuous fundamental frequency (called “pitch trace”) of each syllable rendition. As can be clearly seen on the examples plotted on Fig. 3A (breeding season) and B (early fall), and from the superimposed pitch traces in both conditions (Fig. 3C), the fluctuations in pitch between renditions of the same syllable are largely increased in early fall. This was observed for most syllable types. The CV of the pitch traces (see Methods for details) was significantly increased in early fall songs when comparing the CV of all syllable types from all birds in both conditions ($n = 93$ syllable types in the breeding season: 0.03 ± 0.02 , $n = 64$ syllable types in early fall: 0.07 ± 0.04 , t-test, $t = 6.5$, $p \leq 0.001$, $df = 155$) or the CV of only the syllable types that were sung in both the breeding season and early fall ($n = 48$ syllable types, breeding season: 0.04 ± 0.02 , early fall: 0.06 ± 0.04 , paired t-test, $t = 4.9$, $p \leq 0.001$, $df = 47$, Fig. 3E). When we took into account only syllables ending with a pitch plateau, such as the one shown in Fig. 3A–C, the ending pitch over syllable renditions also showed a much greater variability in early fall ($n = 32$ syllable types, CV of ending pitch in the breeding season: 0.06 ± 0.04 , early fall: 0.09 ± 0.05 , paired t-test, $t = 6.5$, $p \leq 0.001$, $df = 31$, Fig. 3G).

On the contrary, mean pitch was rather stable between the two conditions (Fig. 3D–F). The mean pitch of various syllables types did not change between the breeding season and early fall when all syllable types were considered ($n = 93$ syllable types in the breeding season: 2.9 ± 0.9 kHz, $n = 64$ in early fall: 2.9 ± 0.8 kHz, t-test, $t = 0.18$, $p > 0.05$, $df = 155$). However, a small but significant decrease in the mean frequency was revealed when the same syllable types were compared between the two conditions ($n = 48$ syllable types, breeding season: 3.0 ± 0.9 kHz, early fall: 2.9 ± 0.8 kHz, paired t-test, $t = 2.3$, $p \leq 0.05$, $df = 47$). When focusing on the ending pitch for syllables that showed a stable pitch trace toward the end of the syllable, we found no change in the mean ending pitch of these syllable types ($n = 32$ syllable types sung in both conditions, breeding season: 2.9 ± 0.8 kHz, early fall: 2.8 ± 0.8 kHz, paired t-test, $t = 1.3$, $p > 0.05$, $df = 31$; for all syllable types, $n = 58$ in breeding season: 2.9 ± 1 kHz, $n = 37$ in early fall: 2.9 ± 0.8 kHz, t-test, $t = 1.3$, $p > 0.05$, $df = 93$). These results therefore demonstrate that season dramatically affects variability in acoustic features of syllables while leaving their mean characteristics mostly unchanged.

We also assessed whether the season also had an impact on the sequential ordering of phrases within songs. The number of possible transitions between syllable types in song sequences also became more variable in early fall compared to the breeding season (Fig. 4). In particular, we found that both convergent transitions (toward a given syllable category) and

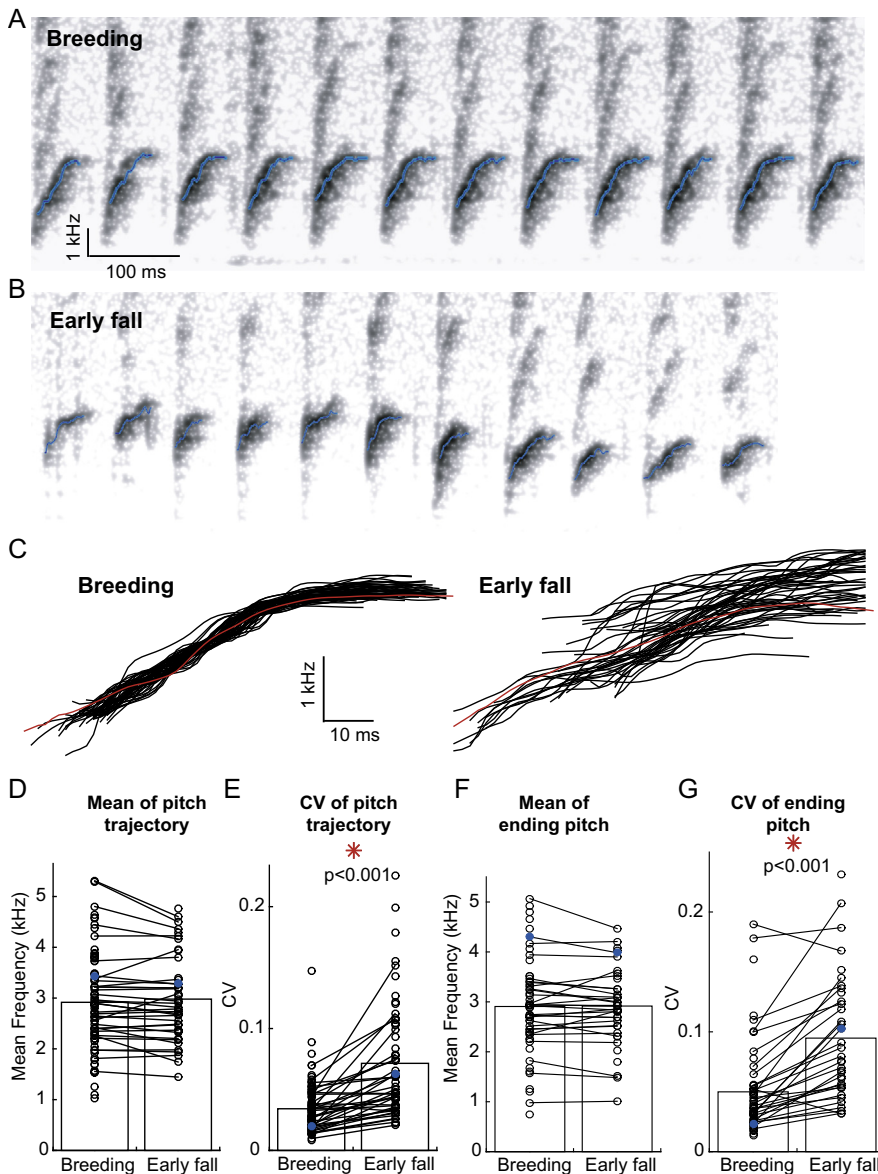


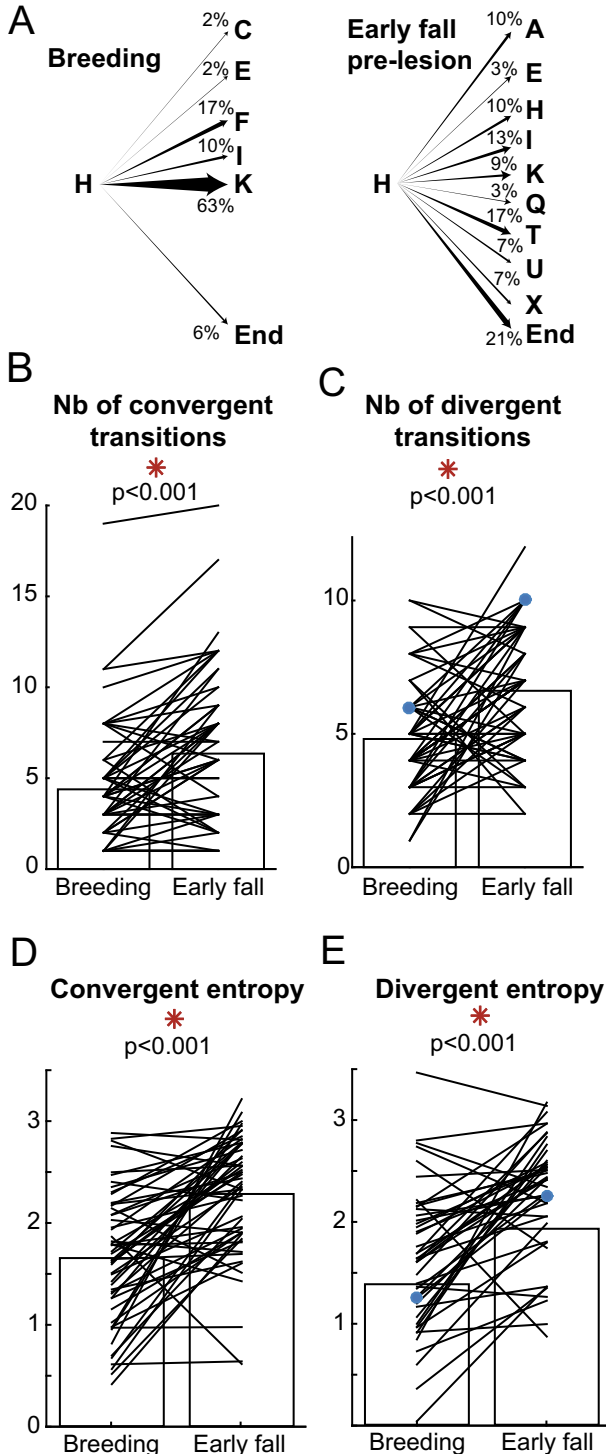
Fig. 3. The acoustics of syllables are more variable in early fall. A) Spectrogram of a single phrase from an example syllable type in breeding season song. Blue lines denote the pitch trajectories extracted for each syllable rendition with the Praat software (see Material and Methods). B) Spectrogram of a single phrase from the same example syllable type as in A, in early fall song. Same conventions and scales as in A. C) Overlaid pitch trajectories from 50 syllable renditions from the syllable type displayed in A and B, in breeding season song (left) and in early fall song (right). Each black trace corresponds to the pitch trace for a single syllable rendition (50 traces shown in each condition for better readability), while the red line denotes the average pitch trace over all renditions considered in a given condition ($n = 630$ and 826 in the breeding season and early fall respectively). Note that pitch trajectories of various renditions have been aligned to reduce the variability induced by time jitter (see Material and Methods). D) Mean frequency of the pitch traces over all syllable renditions in the two conditions for all syllable types displaying at least 10 renditions (analyses were performed on syllable types that were repeated at least ten times, see Material and Methods). In D–G, black lines connect similar syllable types, when present in both conditions, and blue disks highlight the data from the example syllable type shown in A–C. The mean frequency of syllables did not vary from the breeding season to early fall. E) Coefficient of variation (CV) over all syllable renditions, averaged over the pitch trajectories (see Material and methods) in each condition for all syllable types displaying at least 10 renditions. The variability in pitch trajectories of syllables increased strongly in early fall compared to the breeding season. F) Mean ending pitch over all syllable renditions in each condition for all syllable types displaying a stable end in their pitch trace (see Material and Methods). The ending pitch of syllables with a stable end did not vary between the breeding season and early fall. G) Coefficient of variation (CV) of the ending pitch over all syllable renditions, in each condition for all syllable types displaying a stable end in their pitch trace. The variability in ending pitch of syllables increased strongly in early fall compared to the breeding season. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

divergent transitions (from a given syllable category) were more numerous in early fall compared to the breeding season (mean number of convergent transitions over $n = 58$ syllable category, breeding season: 4.6 ± 3.2 , early fall: 6.4 ± 4.1 , paired t-test, $t = 2.57$, $p \leq 0.01$, $df = 57$; mean number of divergent transitions over $n = 58$ syllable category, breeding season: 4.9 ± 2.4 , early fall: 6.8 ± 2.4 , paired t-test, $t = 5.46$, $p \leq 0.001$, $df = 57$; Fig. 4B–C). Hence, we found that the entropy of convergent and divergent transitions (see Material and Methods) is increased in early fall compared to the breeding season (convergent transitions, $n = 58$, breeding season: 1.4 ± 0.9 , early fall: 1.9 ± 0.9 , paired t-test, $t = 4.93$, $p \leq 0.001$, $df = 57$; divergent transitions, $n = 58$, breeding season: 1.6 ± 0.6 , early fall: 2.3 ± 0.5 , paired t-test, $t = 7.85$, $p \leq 0.001$, $df = 57$; Fig. 4D–E), highlighting the increase in the variability of song sequences in early fall.

Quantification of LMAN lesions

Eleven out of 13 birds whose songs were recorded underwent surgery for electrolytic lesion of LMAN ($n = 7$) or a sham-lesion ($n = 4$, see Methods). Nissl-stained sagittal brain slices were used to quantify the LMAN lesion volume in each brain hemisphere (see Fig. 5A and B). Quantification revealed that LMAN was completely or largely damaged in both brain hemispheres of the LMAN-lesion birds (lesion LMAN volume: $80 \pm 30\%$, $n = 12$ brain hemispheres with histological control, for each bird: 100% in three birds, 75% in two birds, 50% in one bird). Birds with sham-lesion did not show any LMAN damage. We were not able to detect any correlation between the extent of the volume lesion in each bird and the effect of LMAN lesion on song. Additionally, the injection of a retrograde fluorescent tracer in nucleus RA confirmed the loss of LMAN-to-RA projection neurons in 3 lesion birds (Fig. 5C) while the projection from HVC to RA was

unaffected. We also focused on morphology of HVC neurons in sham lesion birds and LMAN lesion birds, but we did not notice any clear difference. While lesions were larger than the normal extent of LMAN, they did not touch other song-related nuclei such as Area X or MMAN.



LMAN lesion brings song parameters closer to those from the breeding season

We first conducted a Principal Component Analysis (PCA, see Material and Methods) on the mean values of all song parameters normalized by their value in early fall before lesion, for early fall LMAN- and sham-lesion birds. This “pre-lesion normalization” was intended to ease visualization and reveal whether LMAN lesion brought song parameters closer to breeding season values. The first and second PCA respectively accounted for 87.5% and 7.7% of the variance in the data. Each of these two first PCA was a relatively even representation of all song parameters (6/8 and 7/8 coefficients > 0.1 for the first and second PCA respectively, and all coefficients < 0.6 for both, in absolute values). The PCA reveals that following LMAN lesion, song parameters remained in a closer proximity to the parameters recorded during the breeding season, while song parameters from sham-lesion birds were segregated into a different region of the parameter space (plotted on Fig. 5D). Although individual variability in various song parameters obscures the global picture, the PCA suggests that LMAN lesion may reverse some seasonal effects undergone by canary song between the breeding season and early fall. This qualitative statement was then tested on all song parameters, leading to the results unfolded below.

LMAN lesions reduce song variability and partially reverse seasonal plasticity in early fall

As mentioned above, early fall songs in canaries are ‘unstable’ (Nottebohm, 1981; Nottebohm et al., 1986) and display higher variability than songs recorded during the breeding season. Here, we assessed whether LMAN is involved in the generation of the acoustic and sequence variability that characterize songs produced by canaries in early fall. In Fig. 6, we depict an example of early fall song before LMAN lesion (Fig. 6A) and after LMAN lesion (Fig. 6B). In this example, acoustic variability of the syllable appears strongly reduced after LMAN lesion, while

Fig. 4. The variability in syllable sequencing is increased in early fall. A) Example transition diagram showing the various syllable categories that may follow syllable category H in an example bird, in the breeding season (left) and in early fall (right). The ratio of transitions leading to a given syllable category is indicated above the corresponding arrow. Note the larger number of possible syllable categories following H in early fall compared to the breeding season, and the increased uncertainty about the following syllable category. B) The number of possible syllable categories preceding a given syllable category (convergent transition) is increased in early fall compared to the breeding season. C) The number of possible syllable categories following a given syllable category (divergent transition) is also increased in early fall compared to the breeding season. D-E) Due in part to the increased number of possible transitions, the entropy of both convergent (D) and divergent (E) transitions is increased in early fall compared to the breeding season. C, E) Blue disks indicate the corresponding data of the example transitions shown in A. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

phrase duration is increased, changing the ‘unstable’ early fall song into something much closer to the stereotyped breeding season song. As we will show below, LMAN lesions in early fall at least partially reversed the changes undergone by song in early fall as compared to the breeding season. The repertoire size, which increased in early fall due to the sometime very variable and ‘unsta-

ble’ vocalizations recorded, tended to be reduced by LMAN lesion (Fig. 6C). However, when considering syllable categories according to the rough shape of the pitch trace (see Material and Methods), the number of syllable categories remained constant after LMAN or sham lesions (Fig. 6D). Note that we ensure that the ratio of unclassified vocalization remains stable in all conditions (see Methods).

Song duration was clearly affected by LMAN lesions performed at early fall. Indeed, LMAN lesion tended to restore breeding season-like song and phrase durations (example bird on Fig. 7A-D). In contrast, these song features did not change in the example sham-lesion bird. LMAN lesions in early fall induced a significant increase in song duration in all four birds with lesion (average song duration before lesion: 3 ± 0.5 s, after lesion: 6.4 ± 1.8 s, see Table A in Appendix for results and statistics in individual birds, Fig. 7E). This increase in song duration was due both to an increase in the number of phrases per song (before lesion: 7 ± 1.6 phrases per song, after lesion: 11.3 ± 3.8 , significant

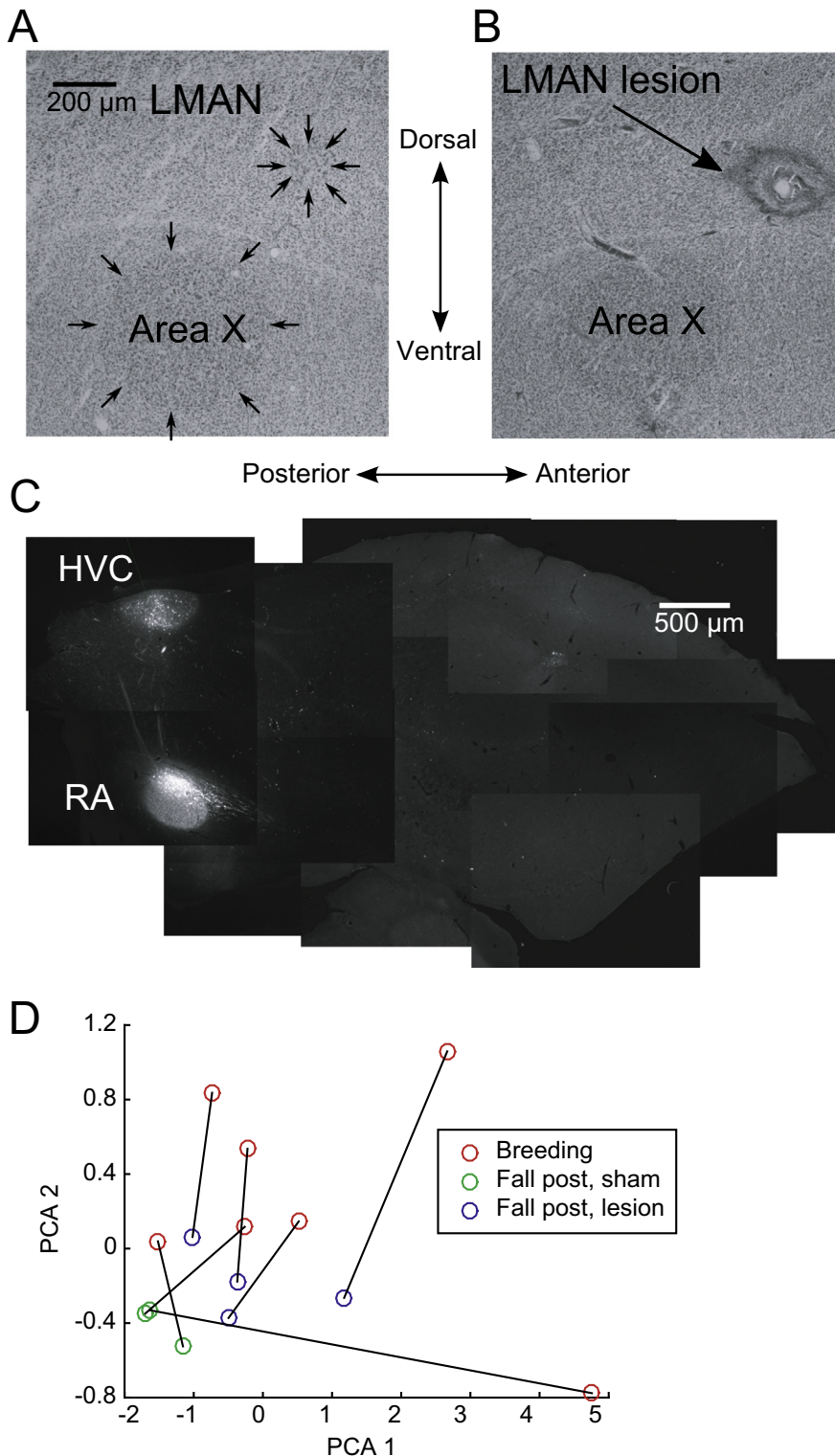


Fig. 5. Histological control of LMAN lesion. A-B) Nissl-stained brain slices in a control bird (A) and in a bird which underwent an LMAN lesion (B). Black arrows delineate the nuclei Area X and LMAN in the control bird. In the lesion bird, the area corresponding to LMAN nucleus displays the burn due to electrolytic lesion. C) Fluorescent markers injected in nucleus RA reveal the extent of LMAN lesion in birds with LMAN lesion. Example sagittal slice (same orientation as A and B) showing injection site in nucleus RA, retrograde tracing in nucleus HVC, while very little retrograde tracing can be seen at the expected location of LMAN, due to the lesion. D) Principal Component Analysis (PCA) from all song parameters extracted quantified in the three different conditions (breeding condition, early fall before lesion and after lesion) for early fall LMAN- and sham-lesion birds: repertoire size, song duration, phrase duration, IPI duration, mean pitch, CV of pitch, entropy of convergent transitions, entropy of divergent transitions. The PCA was run on the averages of each song parameter over each bird in the breeding season and early fall after lesion, divided by the average value in early fall before lesion. The PCA reveals that LMAN lesion brings song parameters in a closer proximity to the parameters recorded during the breeding season, while data from sham-lesion birds were segregated into a different region of the parameter space.

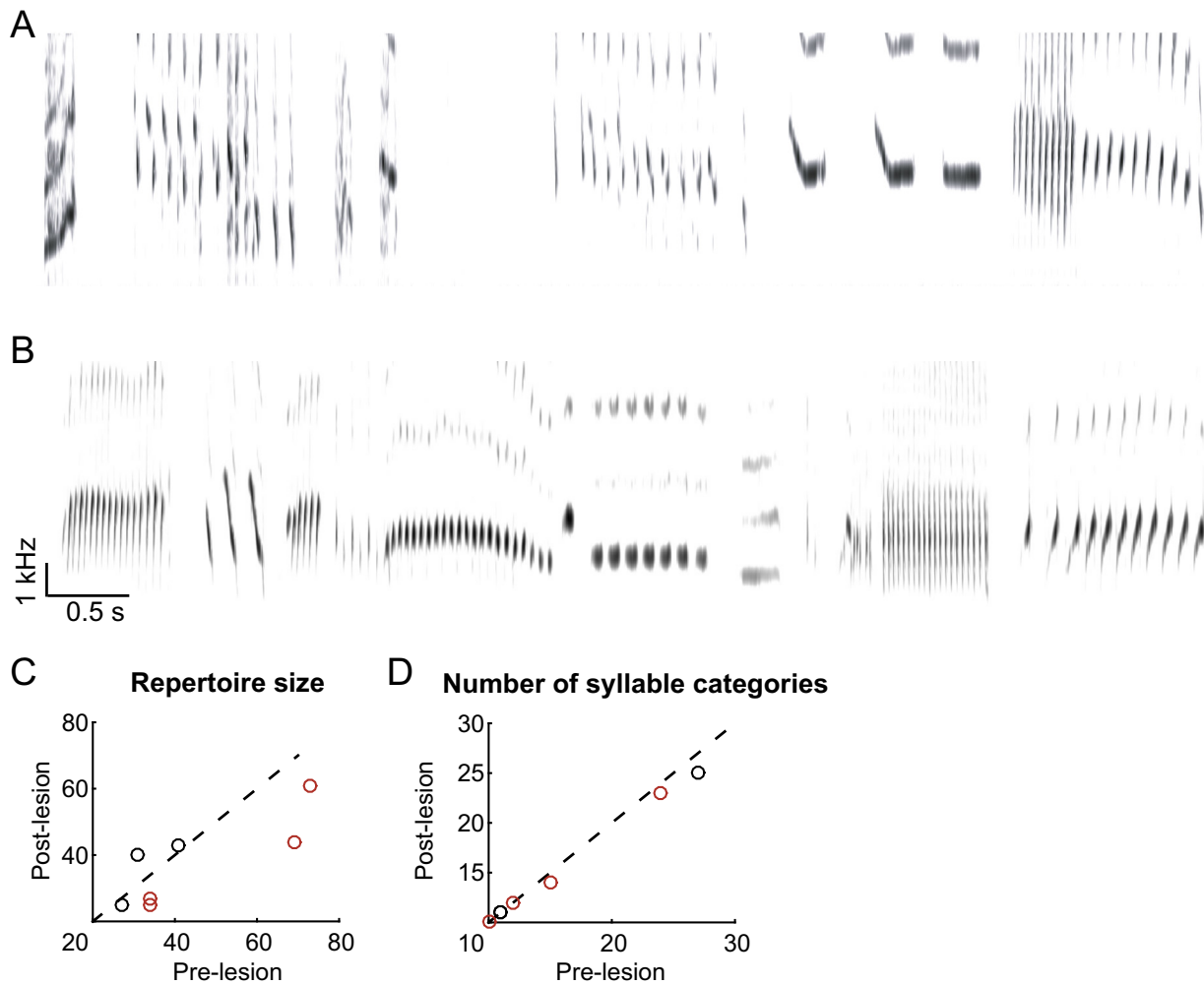


Fig. 6. Songs are more stereotyped following LMAN lesion at early fall. A) Spectrogram of an example canary song recorded in early fall before LMAN lesion. B) Spectrogram of an example song produced by the same bird in early fall following LMAN lesion, in the first days following resuming singing after the lesion surgery. C) Repertoire size in early fall before and after LMAN lesion in all birds. The number of different syllable types is decreased by LMAN lesion. D) The number of syllable categories in early fall remains constant before and after LMAN lesion in all birds. C,D) Black and red open circles denote data from birds that have received a control/sham lesion and LMAN lesion, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increase in three out of four birds, see [Table A](#) in Appendix, [Fig. 7F](#)) and an increase in the duration of song phrases (before lesion: 0.4 ± 0.2 s, after lesion 0.5 ± 0.2 s, significantly increased phrase duration in all four birds, see [Table A](#) in Appendix, [Fig. 7G,H](#)). In addition, IPI tended to be reduced after LMAN lesion, with a significant decrease in only two birds (before lesion: 0.21 ± 0.05 s, after lesion: 0.14 ± 0.04 s, significant decrease within two subjects, see [Table A](#) in Appendix, [Fig. 7I](#)). In contrast, sham lesions in early fall (time-matched to the real lesions) did not induce any consistent change in song duration (before sham-lesion: 5 ± 3 s, after sham-lesion: 5 ± 3 s, see [Table A](#) in Appendix for results and statistics in individual birds, [Fig. 6E](#)), phrase number (before sham-lesion: 9 ± 5 phrases per song, after sham-lesion: 9 ± 4 phrases per song, see [Table A](#) in Appendix for results and statistics in individual birds, [Fig. 7F](#)), phrase duration ($n = 15$ syllable types, before sham lesion: 0.6 ± 0.2 s, after

sham-lesion: 0.6 ± 0.3 s; paired t-test, $t = 0.62$, $p > 0.05$, $df = 14$; see [Table A](#) in Appendix for results and statistics in individual birds, [Fig. 7G,H](#)), nor in IPI (before sham lesion: 0.17 ± 0.03 s, after sham-lesion: 0.15 ± 0.03 s, see [Table A](#) in Appendix for results and statistics in individual birds, [Fig. 7I](#)). We conclude that LMAN exerts a strong influence on song duration at early fall, and that the changes in song, phrase and IPI duration observed at early fall with respect to breeding season song are likely to be due to the increased influence of LMAN on early fall song.

LMAN lesions also had a strong impact at the local level on individual syllables. The acoustic variability of syllables, which dramatically increased in early fall songs compared to the breeding season ([Fig. 3](#)), was indeed clearly reduced by LMAN lesions, reverting the effect of seasonal plasticity ([Fig. 8](#)). As an example, [Fig. 8A-C](#) depicts the frequency trajectories of 50 renditions of one syllable type in the breeding season

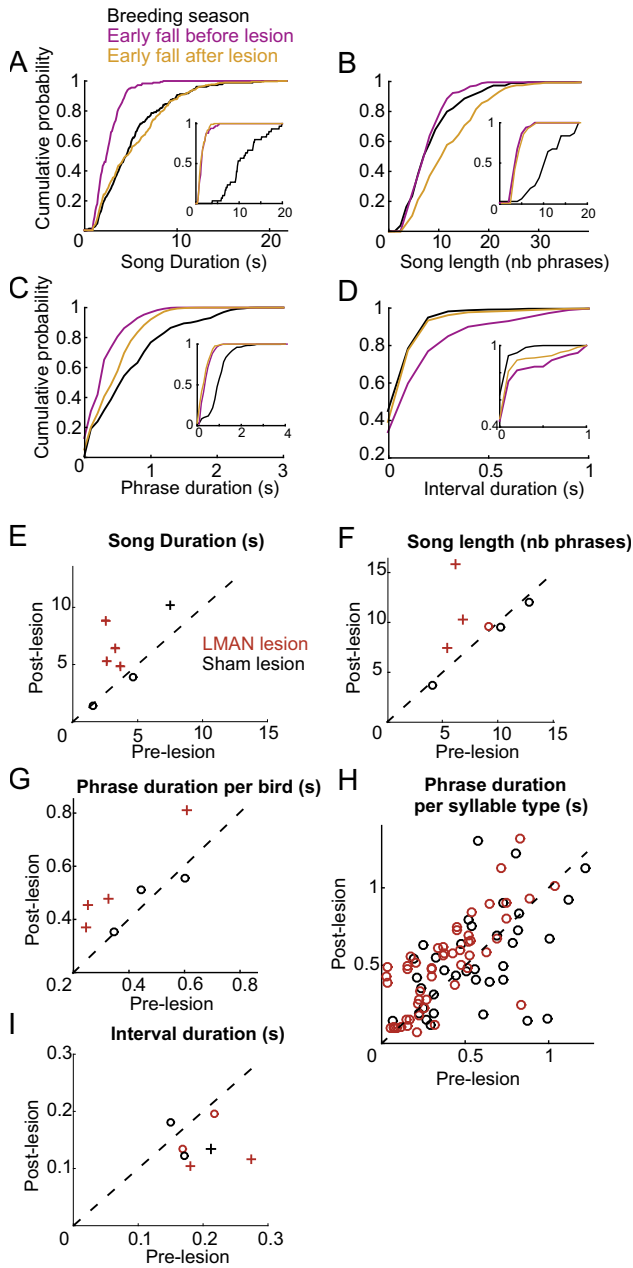


Fig. 7. Lesion of nucleus LMAN in early fall reverses seasonal effects on song and phrase duration. A-D) Reversion of seasonal effects in song duration (A), song length (B), phrase duration (C) and IPI duration (D) by LMAN lesion in the example bird B9 (same as in Fig. 1, main graphs), and but not by sham-lesion in another example bird (insets). In each graph, the cumulative probability of the song feature is represented by black lines in the breeding season, purple lines in early fall before lesion, and in orange after lesion. E-I) Effect of LMAN lesion in early fall on song duration (E), song length (F), phrase duration (G and H), and IP duration (I) for all subjects. In E-G and I, red (resp. black) symbols represent the average data from a single subject which underwent LMAN (resp. sham) lesion before (X-axis) and after (Y-axis) the lesion. Crosses (resp. circles) denote a significant change (resp. no significant change) between the pre- and post-lesion conditions. The dotted line follows the X = Y axis. In H, each circle represents the data from all phrases/syllables of a given syllable type before (X-axis) and after (Y-axis) LMAN lesion (or time-matched period in controls). Red circles denote phrases/syllables from LMAN lesion birds, while black circles denote phrases/syllables from sham-lesion birds. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 8A), early fall before LMAN lesion (Fig. 8B) and after lesion (Fig. 8C). The increased variability in the pitch trace observed in early fall was clearly reduced after LMAN lesion, reverting to breeding season-like variability. Over all syllable types in early fall songs, we found that the variability of the pitch traces was substantially reduced after LMAN lesions. Overall syllable types in birds with a lesion at early fall, the CV of the pitch traces was reduced after lesion ($n = 41$ syllable categories before lesion: 0.07 ± 0.04 , $n = 41$ syllable categories after lesion: 0.05 ± 0.03 , t-test, $t = 2.77$, $p \leq 0.01$, $df = 81$, Fig. 8F) or for the syllable types sung both before and after lesion ($n = 34$ syllable types, before lesion: 0.07 ± 0.04 , after lesion: 0.05 ± 0.04 , paired t-test, $t = 3.4$, $p \leq 0.01$, $df = 33$, Fig. 8F). Similarly, when only considering the plateau frequency of syllables with stable ending, we found that variability in frequency was reduced by LMAN lesion in early fall ($n = 17$ syllables types, CV from 0.09 ± 0.05 before lesion to 0.07 ± 0.05 after lesion, paired t-test, $t = 2.83$, $p = 0.01$, $df = 16$, Fig. 8G). Meanwhile, the average frequency over these pitch traces did not change significantly after lesion ($n = 38$ syllable types before lesion: 2.8 ± 0.7 kHz, $n = 43$ syllable types after lesion: 2.6 ± 0.7 kHz, t-test, $t = 1.1$, $p > 0.05$, $df = 79$; $n = 29$ syllable types sung under both conditions 2.7 ± 0.6 kHz vs 2.7 ± 0.7 kHz, paired t-test, $t = 0.08$, $p > 0.05$, $df = 28$; Fig. 8D), nor did the mean ending pitch in stable ending syllable types ($n = 17$ syllable types, before lesion: 2.5 ± 0.6 kHz, after lesion: 2.5 ± 0.7 kHz, paired t-test, $t = 0.61$, $p > 0.05$, $df = 16$; Fig. 8E). Among sham lesion birds, we found no significant change in the variability or mean of syllable pitch traces ($n = 21$ syllable types, CV before lesion: 0.06 ± 0.05 , CV after lesion: 0.06 ± 0.04 , $t = 0.19$, $p > 0.05$; mean before lesion: 3.1 ± 0.9 kHz, mean after lesion: 3.2 ± 0.9 kHz, $t = -0.81$, $p > 0.05$; both paired t-tests with $df = 20$; Fig. 8D-F). Similarly, the plateau frequency of syllable with stable ending did not change in sham lesion birds ($n = 11$ syllable types, CV before lesion: 0.09 ± 0.05 , CV after lesion: 0.08 ± 0.03 , $t = 1$, $p > 0.05$; mean before lesion: 3.2 ± 1 kHz, mean after lesion: 3.3 ± 1 kHz, $t = -0.28$, $p > 0.05$; both paired t-tests with $df = 10$; Fig. 8E-G). We conclude that LMAN lesion reduced the acoustic variability of syllables produced in early fall, reversing at least partially the seasonal increase in acoustic variability as compared with the breeding season.

Finally, we assessed the effect of LMAN lesion on syllable sequencing in early fall songs. As shown on an example transition (Fig. 9A), LMAN lesion tended to bring back transition probabilities closer to what they were in the breeding season. In particular, the entropy of transitions, which was increased in early fall as compared to the breeding season, was strongly reduced by LMAN lesion. This was true for convergent transitions (among $n = 37$ frequent transitions: before lesion: 2.4 ± 0.5 , after lesion: 2.1 ± 0.6 , 3-way ANOVA, $F = 4.83$, $p \leq 0.01$, $df = 108$, post hoc Tukey's test, $p \leq 0.01$, Fig. 9B), and for divergent transitions (among $n = 37$ frequent transitions, before lesion: 2.1 ± 0.4

after lesion: 1.7 ± 0.6 , 3-way ANOVA, $F = 16.5$, $p \leq 0.001$, $df = 108$, post hoc Tukey's test, $p \leq 0.05$, Fig. 9C). On the contrary, sham-lesion birds did not display any significant change in the entropy of their convergent (among $n = 21$ frequent transitions, before sham-lesion: 2.0 ± 0.4 , after sham-lesion: 2.0 ± 0.5 , 3-way ANOVA, $F = 3.54$, $p \leq 0.05$, $df = 60$, post hoc Tukey's test, $p > 0.05$, Fig. 9B) or divergent transitions (among $n = 21$ frequent transitions, before sham-lesion: 1.8 ± 0.4 , after sham-lesion: 1.8 ± 0.5 , 3-way ANOVA, $F = 4.05$, $p \leq 0.05$, $df = 60$, post hoc Tukey's test, $p > 0.05$, Fig. 9C). Therefore, we can conclude that LMAN lesion reduced the entropy of transitions between syllables in song sequences and moved the song

sequence properties of early fall song closer to that of the breeding season song.

LMAN lesions do not affect songs in the breeding season

Finally, to make sure that the effect of LMAN lesion that we observed in early fall was reflecting an increased influence of LMAN in early fall, driving a seasonal increase in song variability, we assessed whether LMAN lesions had any effect on song during the breeding season, when the song is most stereotyped (Fig. 10). The changes observed after LMAN lesion ($n = 3$) or in control birds ($n = 3$; 1 bird with a sham lesion and 2 birds that did not undergo surgery) in the breeding season were never consistent among subjects, leading to no significant change between the groups. Song duration did not change consistently in birds that received a LMAN targeted lesion ($n = 3$, before

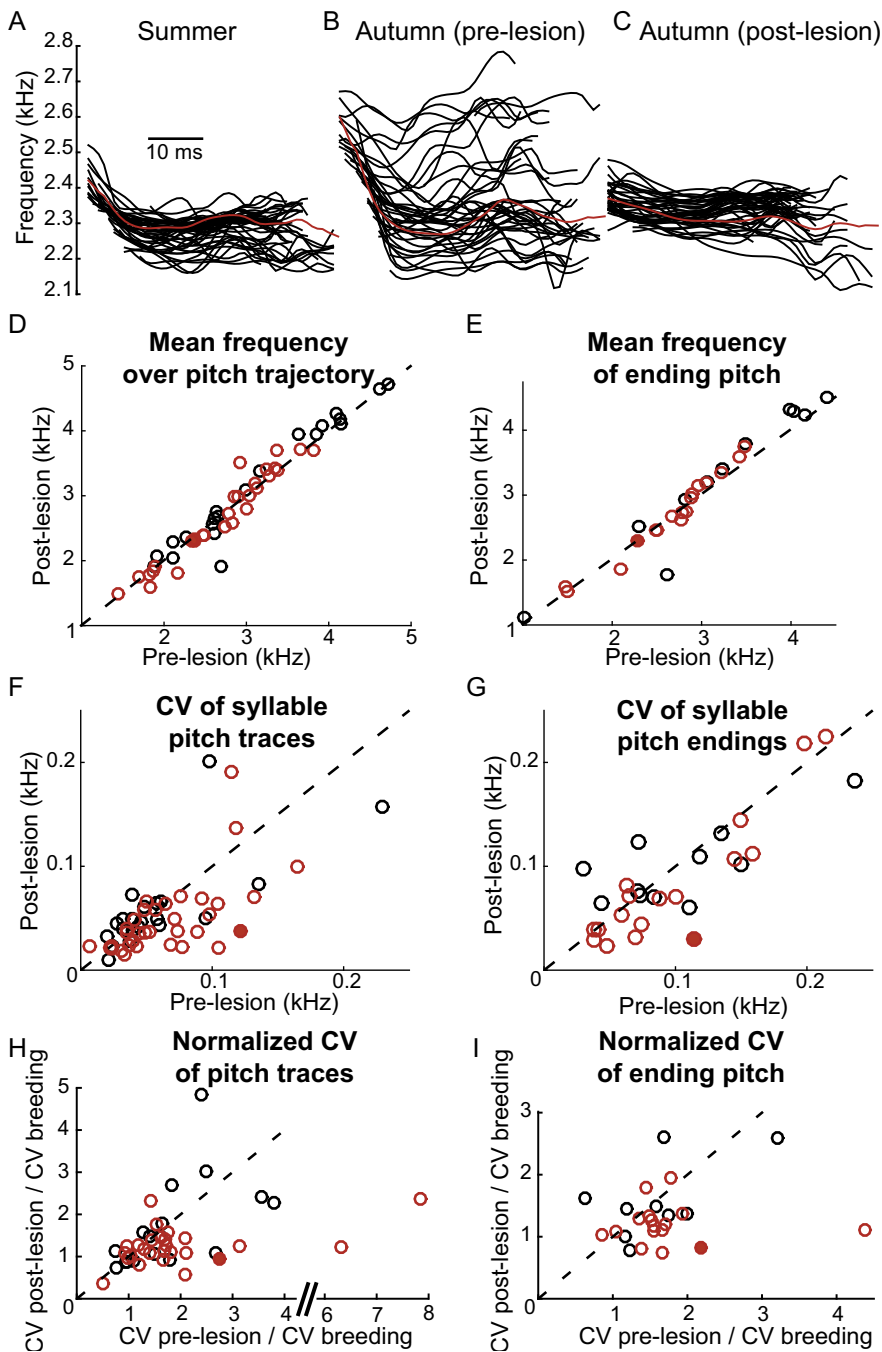


Fig. 8. Lesion of nucleus LMAN in early fall reduces the variability of syllable's acoustics, partially reversing seasonal changes. A-C) Example pitch traces from 50 renditions within an example syllable type in the breeding season (A), early fall before LMAN lesion (B) and after LMAN lesion (C). Note the large increase in the variability of pitch traces from rendition to rendition between the breeding season and early fall, which is reversed by LMAN lesion. D-I) Mean and CV of pitch traces and ending pitch for all syllable types in LMAN and sham-lesion birds. In D-G, each circle represents the data from all phrases/syllables of a given syllable type before (X-axis) and after (Y-axis) LMAN or sham-lesion. Red circles denote phrases/syllables from LMAN lesion birds, while black circles denote phrases/syllables from sham-lesion birds. D-E) The mean pitch (D) and mean ending pitch (E, see Methods) among all considered syllable types is not affected by LMAN lesion in early fall. E) The mean ending pitch of syllables is not affected by LMAN lesion in early fall. F-G) The CV of pitch traces (F, see Methods) and the CV of ending pitch (G) are reduced by LMAN lesion in early fall among all considered syllable types. H-I) LMAN lesion reverses the seasonal effect on pitch variability. In H (resp. I), the X axis represents the ratio between the CV of pitch traces (resp. ending pitch) before LMAN lesion and its CV in the breeding season, while the Y axis represents the ratio between the CV of pitch traces (resp. ending pitch) after LMAN lesion and its CV in the breeding season. Each circle represents the data from all phrases/syllables of a given syllable type in LMAN lesion birds (red) or sham-lesion birds (black). Note that post-lesion ratios are distributed around 1, and the syllable types that underwent the largest seasonal increase in variability also display the largest effect of LMAN lesion. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

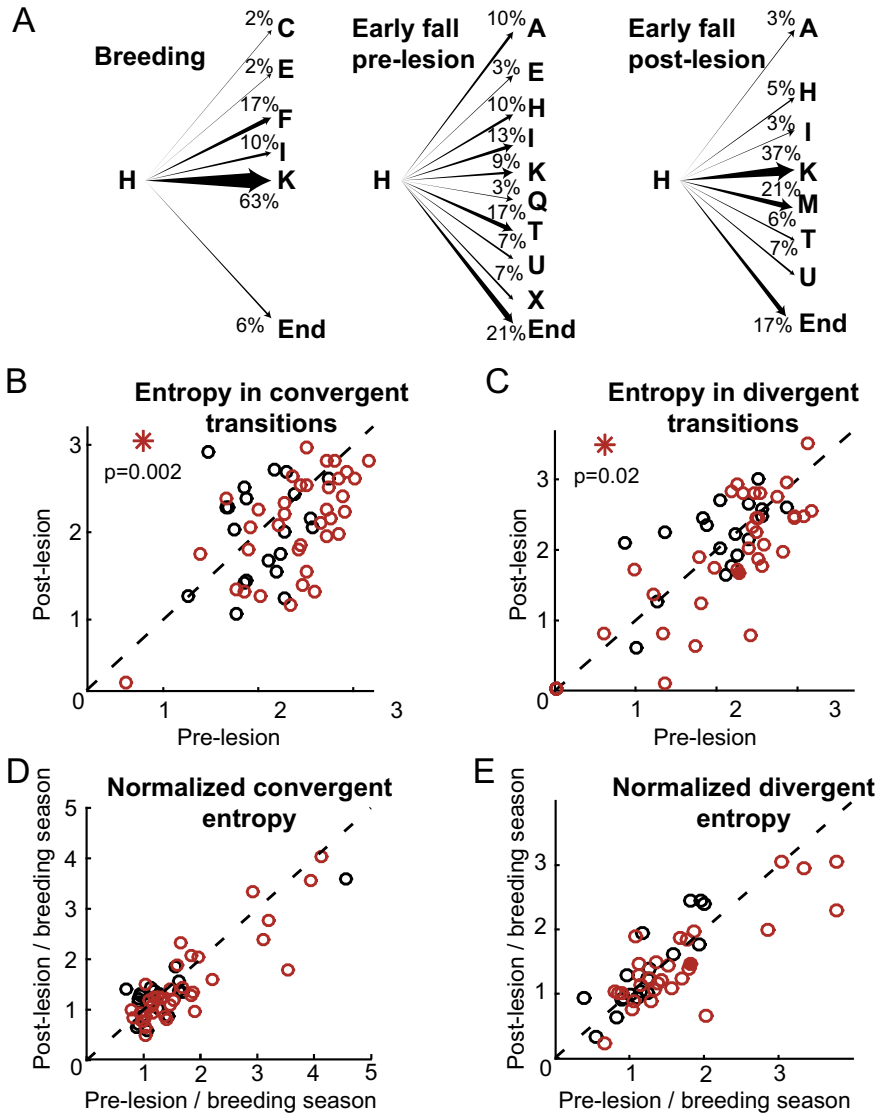


Fig. 9. Lesion of nucleus LMAN in early fall reduces variability in song sequencing. A) Example transition diagram showing the various syllable categories that may follow syllable category H (same example as in Fig. 3) in an example bird, in the breeding season (left), early fall before lesion (center) and after LMAN lesion (right). The ratio of transitions leading to a given syllable category is indicated above the corresponding arrow. Note the decreased uncertainty about the following syllable category following LMAN lesion (few syllable types with higher probability of transition). In B–E, each circle represents the data from all phrases/syllables of a given syllable type in LMAN lesion birds (red) or sham-lesion birds (black). B) The entropy of convergent transitions is reduced following LMAN lesion, while unaffected by sham lesion. C) The entropy of divergent transitions is reduced following LMAN lesion, while unaffected by sham lesion. D–E) Normalized entropy of convergent (D) and divergent (E) transitions before and after LMAN or sham-lesions with respect to the breeding season. While entropy is reduced by LMAN lesion in both cases, the lesion is only partially reverting seasonal increase in transition entropy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lesion: 13.8 ± 2.2 s, after lesion: 13.5 ± 4.6 s, see Table B in Appendix for results and statistics in individual birds, Fig. 10A) as for controls ($n = 3$, before lesion: 12.1 ± 3.8 s, after lesion: 10.1 ± 6.2 s, see Table B in Appendix for results and statistics in individual birds). Phrase duration did not vary in all birds after LMAN lesion ($n = 38$ syllable types, before lesion:

1.3 ± 0.5 s, after lesion: 1.4 ± 0.7 s, paired t-test, $t = 0.89$, $p > 0.05$, $df = 37$; see Table B in Appendix for results and statistics in individual birds, Fig. 10C,D), nor in controls ($n = 35$ syllable types, before lesion: 1.2 ± 0.5 s, after lesion: 1.3 ± 0.7 s, paired t-test, $t = 1.38$, $p > 0.05$, $df = 34$; see Table B in Appendix for results and statistics in individual birds). Maybe more importantly, we did not find any change in the acoustic variability of syllables after LMAN lesion in the breeding season. Indeed, the CV of pitch traces remained unchanged ($n = 38$ syllable types, before lesion: 0.03 ± 0.01 , after lesion: 0.04 ± 0.02 , t-test, $t = 0.26$, $p > 0.05$, $df = 37$, Fig. 10D), as did the mean frequency over these traces ($n = 38$ syllable types, before lesion: 3.4 ± 0.9 kHz, after lesion: 3.3 ± 0.9 kHz, t-test, $t = 1.48$, $p > 0.05$, $df = 37$, Fig. 10E). Similarly, in control birds, the CV and mean of pitch traces did not change (CV among $n = 35$ syllable types, before lesion: 0.03 ± 0.02 , after lesion: 0.04 ± 0.02 , paired t-test, $t = 1.26$, $p > 0.05$, $df = 34$; Mean among $n = 35$ syllable types, before lesion: 3.1 ± 1.3 kHz, after lesion: 3.1 ± 1.1 kHz, paired t-test, $t = 0.16$, $p = 0.8$, $df = 34$). Looking at the ending frequency plateau of syllables did not yield any more change after lesion (results not shown). Overall, there was no more change in the songs of birds undergoing an LMAN lesion in the breeding season than in the control birds. We therefore conclude that LMAN does not have much influence on the highly stereotyped song of canaries during the breeding season.

DISCUSSION

We report here that the output nucleus LMAN of the song-related BG–forebrain circuit is essential for the expression of seasonal changes undergone by canary song in early fall, namely its increased variability and decreased duration compared to the breeding season song. Indeed, LMAN lesions performed in early fall induced a season-specific decrease in song acoustic and syntactic variability and an increase in song and phrase duration, reverting to a great extent observed seasonal changes. Our findings suggest that adult

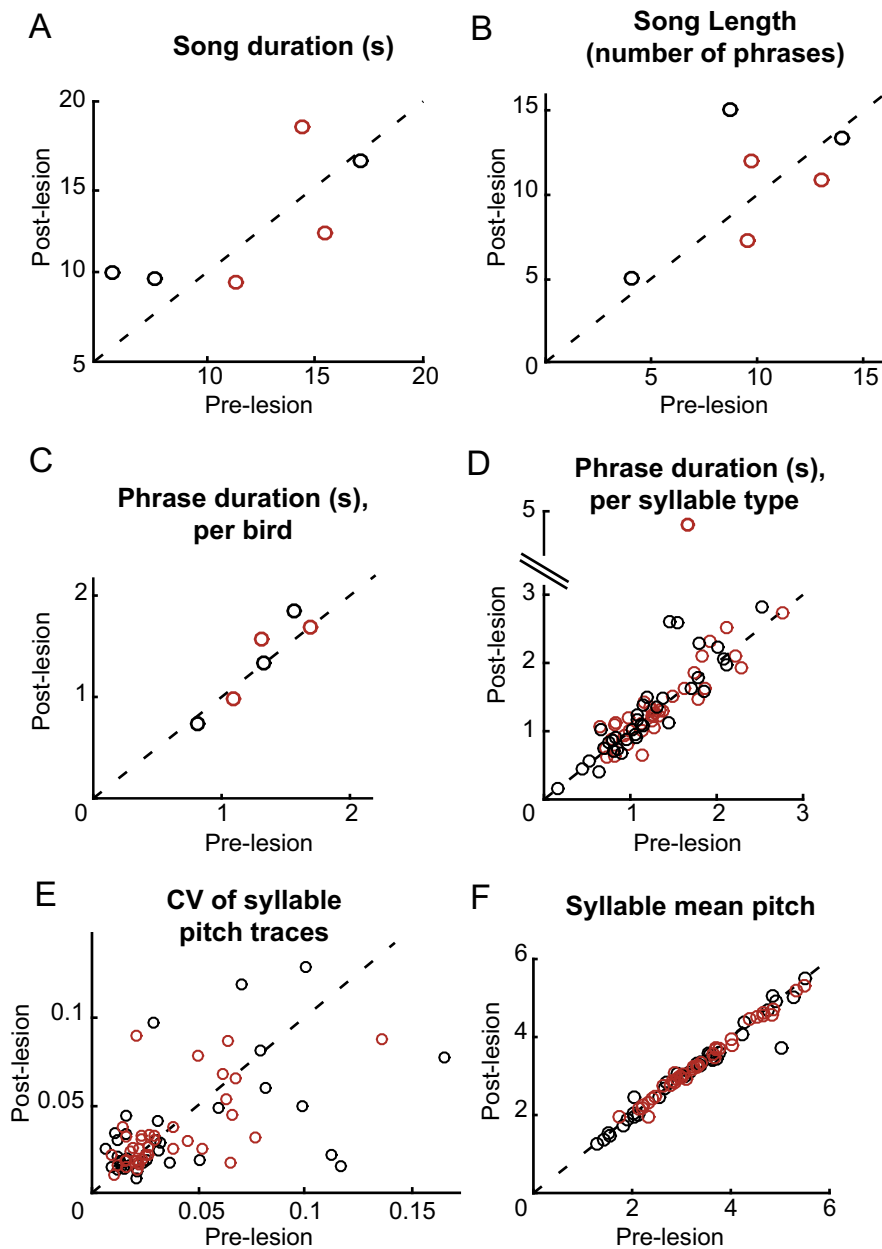


Fig. 10. Lesion of nucleus LMAN in the breeding season does not affect song. A) LMAN lesion does not induce a consistent change in song duration during the breeding season. In A–C, red symbols represent the average data from a single subject which underwent LMAN lesion during the breeding season before (X-axis) and after (Y-axis) LMAN lesion, while black symbols represent the data from time-matched controls. The dotted line follows the $x = y$ axis. B) LMAN lesion does not induce a consistent change in song length (number of phrases per song) during the breeding season. C) Phrase duration is not affected by LMAN lesion, either among or within subjects. D) Over all syllable types considered, phrase duration does not vary after LMAN lesion in the breeding season. In D–F, each circle represents the data from all phrases/syllables of a given syllable type before (X-axis) and after (Y-axis) LMAN lesion or time-matched period in controls. Red circles denote phrases/syllables from LMAN lesion birds, while black circles denote phrases/syllables from control birds. E) The variability of pitch traces is not affected by LMAN lesion in the breeding season. F) The average pitch of syllables is not affected by LMAN lesion in the breeding season. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

seasonal plasticity relies on similar circuits as early ontogeny in juvenile learning, and may thereby exploits similar neural mechanisms, as suggested by Ramon y Cajal (1959).

Cortico–BG circuits are believed to implement skill acquisition through reinforcement learning in mammals and birds (Hikosaka et al., 2002; Graybiel, 2005; Fee and Goldberg, 2011). In particular, the avian BG–forebrain circuit generates song variability in young birds (Olviczky et al., 2005; Aronov et al., 2008). Such motor variability, also referred to as exploration in the reinforcement learning framework, is critical for learning motor skills by trial-and-error (Sutton and Barto, 1998; Tumer and Brainard, 2007; Wu et al., 2014). The ability to learn new songs is assumed to persist throughout life in canaries (Marler and Waser, 1997; Nottebohm et al., 1986, 1987; Mundinger, 1995). In early fall, the observed increase in syllable acoustic variability accompanies the emergence of new syllable types (Alvarez-Buylla and Kirm, 1997). New syllable types may appear through the diversification of old syllable types and gradual splitting into new ones (Nottebohm et al., 1986; personal observation), a process already described during juvenile learning in finches (Tchernichovski et al., 2001). While the new syllables could be part of the syllable type memorized from a tutor during early sensory learning as juvenile, they may also ultimately result from a copying process that would allow adult canaries to modify their repertoire and adjust their songs to those of other individuals in a changing social environment (Lehongre et al., 2009). Our findings that vocal variability expressed in early fall is driven by the same BG–forebrain circuits as in juvenile learning support the hypothesis that seasonally increased song variability may be exploited to facilitate adult learning. Interestingly, LMAN is necessary for testosterone-induced sensorimotor song development in female canaries (Rouse and Ball, 2016), a process leading to song masculinization. In this paradigm, LMAN lesion reduced both song variability and syllable diversity, suggesting a causal role of LMAN-driven variability for adding new syllable types.

In opportunistic breeders, song variability generated by LMAN throughout the year is thought to be involved in the maintenance of a stable song pattern (Brainard

Table B. Data from single birds from the LMAN- and sham-lesion groups in the breeding season. The temporal parameters of song (song duration, phrase duration) are averaged within each subject and condition, to reveal significant effects of lesion. For each case, a Student t-test is run to reveal specific effects of lesion

Parameter	Bird	Breeding pre		Breeding post		Test	t	p-Value
		Sample size	Value	Sample size	Value			
Song duration	Sham 1	90	8 ± 4 s	90	10 ± 6 s	Unpaired t-test	2.9	0.004
	Sham 2	31	6 ± 2 s	61	10 ± 3 s		6.7	3 × 10 ⁻⁵
	Sham 3	38	17 ± 3 s	47	16 ± 4 s		0.26	0.8
	Lesion 1	67	14 ± 10 s	68	19 ± 10 s		2.3	0.02
	Lesion 2	87	15 ± 7 s	80	12 ± 7 s		2.9	0.005
	Lesion 3	45	11 ± 5 s	46	10 ± 4 s		1.8	0.07
Phrase duration	Sham 1	11	1.6 ± 0.5 s	13	1.9 ± 0.7 s	Unpaired t-test	1.15	0.26
	Sham 2	14	0.8 ± 0.4 s	12	0.7 ± 0.4 s		0.45	0.7
	Sham 3	11	1.3 ± 0.5 s	15	1.4 ± 0.4 s		0.14	0.9
	Lesion 1	10	1.7 ± 0.5 s	9	1.7 ± 0.6 s		0.09	0.9
	Lesion 2	14	1.1 ± 0.4 s	14	1 ± 0.4 s		0.59	0.6
	Lesion 3	15	1.3 ± 0.4 s	14	1.6 ± 1 s		0.96	0.3

and Doupe, 2000) and can be modulated by social context to increase song stereotypy in the presence of a potential mate (Kao et al., 2005). Increased acoustic variability in fall may also benefit the maintenance of stable syllable types even in close-ended seasonal species (e.g. white-crowned sparrow, Brenowitz, 1997) by contributing in a season-specific manner to motor exploration. In this view, seasonally modulated variability could allow strong plasticity outside the breeding season and optimal stereotyped production when mating is desired in temperate species. Consistently, LMAN lesion outside the breeding season results in unstable song pattern in the following breeding season (Benton et al., 1998).

Alternatively, seasonal plasticity in the avian brain may represent a compromise between the need to continue to form and store new memories throughout the relatively long lifespans of songbirds, and the pronounced size and energetic constraints on the brain imposed by the demands of flight (Calder and King 1974; Tramontin and Brenowitz, 2000; Brenowitz and Larson, 2015). In this view, song alteration observed during fall would result from structural changes undergone by the song system to save energy and brain size outside the breeding season, rather than reflect an on-going learning process.

While acoustic variability in plastic song is largely driven by LMAN in juvenile finches (Olviczky et al., 2005; Aronov et al., 2008), the role that BG–forebrain circuits play in song sequence variability remains less clear. At first glance, our current understanding of the brain mechanisms underlying song production favors a division of labor between the motor pathway, guiding song sequence and timing through HVC input to RA, and the AFP contributing solely to acoustic modulation of syllables. However, this understanding mainly arises from experiments performed on adult zebra finches, which have a fixed song sequence, and overlooks a number of reported observations. Previous studies indeed reported opposite results depending on the species and age of the subjects. Variability in syllable sequencing is reduced following lesion or inactivation of LMAN in juvenile zebra finches (Olviczky et al., 2005; Scharff and Nottebohm,

1991), while no changes were observed in adult zebra finches (Bottjer et al., 1984; Scharff and Nottebohm, 1991; Kao and Brainard, 2006) or in adult Bengalese finches (Hampton et al., 2009). Our results in canaries show that sequence variability, increased in early fall, is clearly reduced after LMAN lesion during that season. It suggests that LMAN is crucial for the generation of sequence variability in canaries outside the breeding season. The magnitude of seasonal changes in transition entropy that we observe in canaries (~40%) is substantially greater than the modulation of transition entropy by social context in Bengalese finches (<25%), and may help reveal LMAN role in sequencing variability in adult birds. The lesser influence of LMAN in adult finches as opposed to juveniles may also explain the lack of effect of LMAN lesion on syntactic variability in adult Bengalese finches. Similarly, we found that LMAN does not affect song syntactic variability during the breeding season in canaries, likely due to an overall diminished influence of this nucleus on the song. However, a difference in the role of LMAN between seasonal and opportunistic breeders cannot be excluded.

Beyond a possible effect of LMAN in the variability of sequence transition, we show here that LMAN lesion can affect the global structure of songs (branching transition sequence and duration). In particular, song length was strongly modulated seasonally and affected by LMAN lesion. This result is in sharp contrast with the dichotomy in finches between the functions of the motor pathway controlling song sequence, tempo and duration, and the BG–forebrain loop controlling acoustic variability (Kao et al., 2005; Long and Fee, 2008; Ali et al., 2013). However, it is difficult to make a parallel between these apparently conflicting results. Indeed, previous results were obtained in zebra finches whose song motif consists of a stereotyped sequence of syllables and varies only due to elasticity in gap and syllable duration (Glaze and Troyer, 2006). On the contrary, phrase duration in canaries relies on the number of repetitions of a single syllable type, and the duration of a song varies due to the duration of its phrases and the number of phrases it contains (depending on the transition

sequence). Therefore, as long as LMAN impacts the sequence transition probabilities, it is expected to influence song duration.

The mechanism by which LMAN influences on song varies with season (in seasonal plasticity) or with age (in juvenile learning) remain to be uncovered. It is interesting to note, however, that, in both cases, induction of large variability in the song by LMAN occurs in the condition where HVC inputs to RA are not as prominent. Indeed, HVC axons enter RA relatively late during development (Konishi and Akutagawa, 1985; Herrmann and Arnold, 1991), only make functional synapses in RA in the early sensorimotor phase of juvenile song learning (Mooney, 1992; Kittelberg and Mooney, 1999), and progressively increase their strength as the song becomes more and more stereotyped (Ölveczky et al., 2011). Similarly, seasonal plasticity gives rise to change in the number of inputs from HVC to RA neurons. First, a large number of neurons projecting to RA is incorporated in HVC in the breeding season (Alvarez-Buylla et al., 1988; Tramontin and Brenowitz, 1999; Scotto-Lomassese et al., 2007). Secondly, the number of dendritic spines increases in RA (Hill and DeVoogd, 1991) likely due to more numerous synapses from HVC onto RA neurons. Meanwhile, neuron number in LMAN and their inputs to RA remain relatively constant (Thompson et al., 2007). Both in juvenile learning and seasonal plasticity, LMAN drives variable patterns of activity in RA, resulting in less structured and more variable songs. As HVC input to RA becomes larger, it drives stronger bursting in RA interleaved with strongly hyperpolarized periods of silence due to recurrent inhibition (Ölveczky et al., 2011). LMAN inputs to RA are mediated mostly through voltage-dependent NMDA receptors (Mooney and Konishi 1991; Ölveczky et al., 2005), and its influence on RA firing is weak in this condition. The influence of LMAN on RA is thus diminished, resulting in a progressively more stereotyped song (Ölveczky et al., 2011). Interestingly, this scenario does not require any seasonal modification in the BG–forebrain circuit to see its influence on song strongly modulated. Structural and molecular seasonal changes in the song-related BG–forebrain circuit may however also contribute to a change in the circuit's influence on song. These include a change in the size of the song-related BG nucleus (Tramontin et al., 2000; Thompson et al., 2007) and a change in the subunit composition of NMDA receptors in LMAN (Singh et al., 2003). LMAN efferent connectivity is mostly mediated by NMDA receptors (Mooney and Konishi 1991; Ölveczky et al., 2005), and changes in their composition may impact neural activity in RA and thereby the song. As testosterone levels that drive increased song stereotypy was shown to accelerate the maturation of intrinsic properties of LMAN projection neurons in juveniles (Livingston and Mooney, 2001), this nucleus may also be a target site for sex steroids triggering seasonal plasticity.

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