Vocal motor changes beyond the sensitive period for song plasticity

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SENSITIVE PERIODS REGULATE the extent of learning and plasticity in a variety of sensory and behavioral systems. For example, the visual system is critically shaped by environmental stimuli during a focal period in development (reviewed in Hensch 2005), and the acquisition of complex behaviors such as language and music are heightened during sensitive periods in human development (Kuhl 2010; Trainor 2005). Sensitive periods are characterized by enhanced levels of plasticity relative to periods preceding the opening and following the closing of the sensitive period (Erzurumlu and Gaspar 2012; Hensch 2005). However, various degrees of plasticity have been noted beyond the closure of the sensitive period for sensory systems such as vision and complex behaviors such as language (Creutzfeldt and Heggelund 1975; Kuhl 2010).

Like speech and language, birdsong is a vocal communication signal that is learned during a sensitive period in development (Doupe and Kuhl 1999). As young fledglings, songbirds such as Bengalese finches memorize the songs of adult tutors and then engage in vocal practice to hone their initially noisy and variable vocalizations into song elements (“syllables”) and sequences that resemble those memorized from adults (Brainard and Doupe 2000, 2002; reviewed in Doupe and Kuhl 1999; Immelmann 1969; Mooney 2009; Price 1979; Tchernichovski et al. 2001). To accurately develop a species-typical song, juvenile songbirds must hear and memorize song during a sensitive window in development (“sensory phase”) and engage in vocal practice during a distinct sensitive period (“sensorimotor phase”; Brainard and Doupe 2000, 2002; Mooney 2009; Tschida and Mooney 2012). As juveniles practice their song during the sensorimotor phase, the sequencing, timing, and structure of song syllables become more stereotyped (reviewed in Doupe and Kuhl 1999; Glaze and Troyer 2013; Johnson et al. 2002; Mooney 2009; Ölveczky et al. 2005; Podos et al. 2009; Tchernichovski et al. 2001).

The sensitive period for song plasticity is thought to close around 3–4 mo of age, when songbirds reach sexual matura-
tion (reviewed in Brainard and Doupe 2000, 2002). This notion is supported by the finding that the gross structure of adult song does not dramatically change over time (reviewed in Brainard and Doupe 2002). Furthermore, neural plasticity mechanisms are progressively lost across juvenile development. For example, dendritic spines in HVC (proper name) stabilize and spines in LMAN (lateral magnocellular nucleus of the anterior nidopallium) are pruned following juvenile song learning (Nixdorf-Bergweiler et al. 1995; Roberts et al. 2010). Furthermore, the expression of cellular mediators of plasticity such as NMDA receptors, CAMK2A, FOXP2, and MAP2K1 in brain nuclei important for song learning decline across juvenile development (Aamodt et al. 1992; Balmer et al. 2009; Haesler et al. 2004; Kato and Okanoya 2010).

However, recent experiments demonstrate that adult birdsong retains some plasticity. For example, targeted auditory manipulations drive adaptive plasticity in the sequencing, timing, and structure of song syllables (Ali et al. 2013; Andalman and Fee 2009; Charlesworth et al. 2011, 2012; Tumer and Brainard 2007; Warren et al. 2011, 2012). Furthermore, experimental manipulations of auditory feedback cause acute and plastic changes to adult song (e.g., Brainard and Doupe 2001; Osmanski and Dooling 2009; Sakata and Brainard 2006; Sober and Brainard 2009). These data indicate that vocal plasticity mechanisms persist in the adult songbird brain and have led to the suggestion that adult song should continue to represent a form of vocal practice (reviewed in Brainard and Doupe 2013).

Despite demonstrations that experimental manipulations can drive vocal plasticity, there is limited evidence that adult song naturally changes over time. Natural changes to vocal performance (e.g., increased song consistency) would complement experimental studies and support the notion that plasticity mechanisms are engaged during adult song production. To this end, we analyzed the degree to which the temporal and spectral
structure of adult Bengalese finch song changes over time. Using a repeated-measures design, we compared how the sequencing, timing, and structure of song syllables changed across months and years in adult Bengalese finches. We explicitly tested the hypothesis that adult song, like juvenile song, becomes more stereotyped and consistent with age.

METHODS

Animals and data collection. Bengalese finches were either born and raised in a breeding colony at the University of California, San Francisco (UCSF; n = 10) or purchased from vendors (Exotic Wings and Things, Ontario, Canada; n = 12). Birds purchased from outside vendors were 2–3 mo of age when shipped to McGill University. All birds were housed on a 14:10-h light-dark cycle with other birds in group cages and provided food and water ad libitum. All procedures were approved by the UCSF Institutional Animal Care and Use Committee or by the McGill University Animal Care and Use Committee in accordance with the guidelines of the Canadian Council on Animal Care.

For song recordings, birds were housed individually in sound-attenuating chambers (Acoustic Systems, Austin, TX; TRA Acoustics, Ontario, Canada). Song was recorded using an omnidirectional microphone (Countryman Associates, Menlo Park, CA) positioned above the male's cage. Computerized, song-activated recording systems were used to detect and digitize song (EvTAF, E. Tumer, UCSF, digitized at 32 kHz; or Sound Analysis Pro v.1.04, http://ofer.sci.cuny.edu/html/sound_analysis.html, digitized at 44.1 kHz). Recorded songs were digitally filtered at 0.3–8 kHz for off-line analysis using software custom written in the MATLAB programming language (The MathWorks, Natick, MA).

To analyze the natural vocal motor changes to adult birdsong, the songs of adult male Bengalese finches (n = 22) were recorded at two ages (younger adult vs. older adult) following sexual maturation. Younger adult recordings were conducted when males were, on average, 6 mo old (range: 4–11 mo). At this age, song is relatively stable compared with juvenile song, and new songs cannot be learned in normally reared birds (reviewed in Okanoya 2004). Older adult recordings were conducted when birds were, on average, 23 mo old (range: 11–55 mo). Recordings were separated, on average, by 17 mo (range: 6–47 mo). Between recording sessions, males were housed in either all-male cages (n = 15) or breeding cages (n = 7). The direction and magnitude of vocal motor changes were not affected by housing condition (P > 0.05 for all comparisons); therefore, all males were analyzed without regard to housing. At least 30 songs from random times across a single day were selected and analyzed at each age for all but 2 birds for which only 25 and 22 songs were recorded as older adults. All songs used for the analysis of age-dependent changes were spontaneous songs produced in isolation (“undirected song”).

Bengalese finches were, on average, 2 yr old at the time of the older recording. Because Bengalese finches can live up to 10 yr, our older recordings were not of senescing Bengalese finches. A previous study demonstrated age-related declines in song performance in Bengalese finches that were >6 yr of age (Cooper et al. 2012).

Song analysis. Bengalese finch song consists of acoustic elements arranged in specific sequences. For purposes of description and analysis, we use the term “syllable” to refer to individual acoustic elements that are separated from each other by at least 5 ms of silence (e.g., Okanoya and Yamaguchi 1997). Labeling and analysis of syllables were identical to those of previously published studies (Sakata and Brainard 2006; Sakata et al. 2008; Warren et al. 2012). Briefly, following amplitude-based syllable segmentation (MATLAB), we labeled syllables manually based on visual inspection of spectrograms using custom-written MATLAB software. Bengalese finch song consists of syllables arranged in stereotyped and variable (“branch point”) sequences (Fig. 1A) (Jin 2009; Okanoya 2004; Okanoya and Yamaguchi 1997; Sakata and Brainard 2006; Wohlgemuth et al. 2010; Woolley and Rubel 1997). An example of a branch point sequence is provided in Fig. 1, in which the sequence “abc” can be followed by either “d” or “q”. This variability is not simply biological noise but reflects a controlled aspect of song (e.g., Sakata and Brainard 2006; Sakata et al. 2008; Warren et al. 2012).

To quantify age-dependent changes in syllable sequencing, we analyzed how sequence transition probabilities and transition entropies of branch point sequences changed with age. We analyzed the probability of different syllable transitions immediately following a specific sequence of syllables. Typically, there are two to five transitions at individual branch points. For each branch point, this sequence variability was quantified as the transition entropy:

\[ \text{transition entropy} = \sum_{i} -p_i \log_2(p_i), \]

where the sum is over all possible transitions, and \( p_i \) is the probability of the \( i \)th transition across all songs (Gentner and Hulse 1998; Gil and Slater 2000; Sakata and Brainard 2006; Sakata et al. 2008). Branch points with transitions that are more variable (i.e., closer to uniform probability) have higher transition entropy scores. Sequences in which the most prevalent (i.e., dominant) transition occurred >95% of the time were not considered branch points. Instances in which song was terminated immediately following the branch point were not included in the calculation of entropy. Only branch points that occurred at least 15 times were analyzed (range: 17–442; mean ± SE: 120.9 ± 5.2, e.g., Sakata and Brainard 2009). Age-dependent changes in syllable sequencing within stereotyped sequences were rarely observed and, hence, not analyzed.

We labeled songs from recordings of younger and older adults and identified branch point sequences for each time point independently. We independently identified branch point sequences in younger and older recordings so as not to bias our results (e.g., by analyzing age-dependent changes only in branch points identified from young adult song, one neglects the possibility that stereotyped sequences become variable). We also paid close attention to long range statistics (i.e., history dependence) in Bengalese finch song (e.g., Fujimoto et al. 2011; Katahira et al. 2011; Warren et al. 2012). For example, if a branch point sequence “cd” was preceded either by an “a” or a “b”, we examined whether transition probabilities were different across “acd” and “bcd” sequence contexts. If transition probabilities were significantly different across sequence contexts, we analyzed the sequences separately; otherwise, we pooled across sequence contexts.

Age-dependent changes in song tempo were analyzed using methods comparable to previous studies (Kao and Brainard 2006; Sakata and Brainard 2006, 2009; Sakata et al. 2008). Specifically, we identified sequences that were commonly produced at both time points and measured the duration from the onset of the first syllable of the sequence to the onset of the last syllable in the sequence (“sequence duration”). We computed the mean and variance of these sequence durations. Only one sequence was selected and analyzed per bird, and we used the interquartile range (IQR; the distance between the 25th and 75th percentiles) as our measure of variance because it is robust to outliers (e.g., Leblois et al. 2010; Samuels and Witmer 2002; Thompson et al. 2011). To compare song tempo across ages and recording conditions, we normalized waveforms within each recording. To normalize the data, we extracted the waveform of focal sequences then resampled (1 kHz), smoothed (5-ms square window), and rectified the waveform. Then, we normalized the waveform between 0 and 1 for both the young and older adult recordings. After this normalization, the amplitude traces across ages were comparable, and we used a common threshold on these normalized traces to find syllable onsets and offsets at each age.

Changes to song tempo could be driven by changes to the duration of syllables, the duration of silent periods between syllables (“gaps”), or both (Andalman et al. 2011; Cooper and Goller 2006; Glaze and Troyer 2006, 2013; Long and Fee 2008; Thompson et al. 2011). To
we analyzed changes in the mean and variability of seven other components. Each example of a syllable was visually screened to ensure that only examples devoid of sound artifacts that could affect FF calculations (e.g., sound of movement, female calls in background) were used in the analysis. The FF of syllables varied from rendition to rendition, and transitions of the branch point sequence “cd” were used to improve the resolution of frequency estimates, we performed a parabolic interpolation of the autocorrelation function improves the accuracy of FF measurements, although this can be impacted when background noise is high or if the signal contains strong high-frequency components. Each example of a syllable was visually screened to ensure that only examples devoid of sound artifacts that could affect FF calculations (e.g., sound of movement, female calls in background) were used in the analysis. The FF of syllables varied from rendition to rendition, and we characterized this variation using the IQR (e.g., Leblois et al. 2010; Thompson et al. 2011).

To further investigate age-dependent changes to syllable structure, we analyzed changes in the mean and variability of seven other acoustic features for each syllable in the bird’s repertoire (mean frequency, density of the spectral entropy, syllable duration, loudness entropy, frequency slope, amplitude slope, and spectrotemporal entropy: e.g., Sakata and Brainard 2006).

Statistical analyses. Analyses were done on a syllable or sequence basis using paired t-tests or mixed-effects models. We measured multiple distinct examples of song features (e.g., FFs of multiple syllables, transition entropies of multiple branch points) for a number of Bengalese finches. For paired t-tests, syllables or sequences were considered individually. For the mixed-effects models (e.g., Reed and Kaas 2010), independent variables were age, bird identification (ID), and syllable ID or sequence ID nested within bird ID. Bird ID, syllable ID, and sequence ID were random variables. This model statistically controls for the fact that multiple syllables or sequences from a single individual are present in the data set. The results from the paired t-test and mixed-effects model were comparable, and results from both tests are presented.

For all tests examining age-dependent changes in song, we set \( \alpha = 0.05 \) (2-tailed). Analyses were conducted using JMP 8 (SAS Institute, Cary, NC) and MATLAB.

RESULTS

Stability of adult song over shorter timescales (1–2 mo). The songs of juvenile songbirds change dramatically over the course of 1–2 mo. During juvenile song development, significant changes to the structure, timing, and sequencing of song...
syllables can occur within days, underscoring the heightened levels of plasticity during this period (Deregnacourt et al. 2005; Lipkind et al. 2013; Tchernichovski et al. 2001). In contrast, studies of adult songbirds indicate that song organization changes very little over months for adult Bengalese finches (>3 mo old; e.g., Hampton et al. 2009; Okanoya and Yamaguchi 1997; Sakata and Brainard 2006; Warren et al. 2012; Woolley and Rubel 1997).

To assess the degree to which our population of adult Bengalese finches demonstrated such song stability, we compared the sequencing, timing, and structure of syllables over 1–2 mo when Bengalese finches were 4–6 mo old (n = 12 birds). Consistent with previous findings, we found that the organization of adult Bengalese finch song did not significantly change over 1–2 mo. For example, stereotyped sequences remained stereotyped, and branch point sequences continued to be variable in sequencing. To quantify and compare sequence variability at branch points, we computed the transition entropy of distinct branch point sequences (n = 45) and found no significant change across 1–2 mo (mixed-effects model: \( F_{1,14,91} = 1.85; P = 0.1939 \); paired t-test: \( t_{44} = 1.50, P = 0.1412 \)). We also found that the duration of syllable sequences (paired t-test: \( t_{11} = 1.44, P = 0.1783 \)), mean FF of syllables with flat harmonic structure (mixed-effects model: \( F_{1,10,19} = 0.12; P = 0.7342 \); paired t-test: \( t_{26} = 0.45, P = 0.6577 \)), and the variability of FF did not significantly change over 1–2 mo (mixed-effects model: \( F_{1,7,84} = 0.12; P = 0.7388 \); paired t-test: \( t_{26} = 0.30, P = 0.7665 \)). These lack of significant changes are not due to insufficient statistical power, because comparable sample sizes have been sufficient to detect other experimental effects on song (e.g., Sakata et al. 2008; Sakata and Brainard 2006; Hampton et al. 2009). These data confirm that the songs of our young adult Bengalese finches are stable across a few months.

Vocal motor changes across longer timescales: syllable repertoire and sequencing. Whereas the previous analysis suggests that adult song is stable, it is possible that adult song retains some plasticity after sexual maturation but that the rate of adult vocal motor change is relatively slow compared with the rate of juvenile vocal motor plasticity. Consequently, it is possible that significant changes to adult vocal performance could be observed across longer periods of time. To test this, we compared the songs of young adult Bengalese finches (4–11 mo of age) with the songs produced by these same males 6–47 mo later (mean interval: 17 mo).

Broadly speaking, as observed in previous studies (e.g., Okanoya and Yamaguchi 1997; Sakata and Brainard 2006; Warren et al. 2012; Woolley and Rubel 1997), the gross structure and organization of songs remained stable, even after years (Fig. 1, A and B). For example, the songs of individual males typically consisted of the same syllables across age: 96% (259/269) of the syllables observed in the songs of young adult males continued to be produced as older adults. Some syllables were “lost” or “gained” over time, but these represented very minor changes to syllable repertoire. The 10 syllables that were “pruned” from the songs of younger adults tended to be syllables that were infrequently produced when birds were young adults; on average, pruned syllables constituted 3.1 ± 0.9% (mean ± SE) of the young male’s repertoire. Five syllables were found in the songs of older adult males but not in the songs produced as young adults, and these gained syllables represented 5.9 ± 3.3% of the older male’s repertoire. Therefore, in the rare instances in which the syllable repertoire of a bird changed over time, the magnitude of change was generally small. As such, the syllable repertoire of an adult Bengalese finch is generally stable across years.

In contrast to the stability of adult syllable repertoire, the sequencing and timing of song syllables changed significantly over time. Whereas stereotyped sequences remained the same over time, syllable sequencing at branch points showed consistent changes from young adult to older adult recordings. In particular, syllable sequencing at branch points became more stereotyped as young adults aged into older adults. In the example depicted in Fig. 1, A–C, the branch point sequence “cd” was followed by “b” 11% of the time and by “e” 89% of the time when the bird was 5 mo old. However, 13 mo later, only the transition to “e” was produced following “cd”. In another bird, the branch point syllable “X” was followed by the syllable “a” 2% of the time, “b” 44% of the time, and “c” 54% of the time when the bird was 4 mo old (Fig. 1D). Twenty-five months later, the transitions to “b” and “c” were retained but the transition to “a” was no longer observed. For both of these examples, because the number of possible transitions decreased over time, syllable sequencing became more stereotyped and transition entropy decreased as the birds aged.

Decreases in transition entropy were common across branch point sequences. Of the 98 branch point sequences (in 22 males), 69 branch points (70%) showed decreases in transition entropy over time. Across all branch points, transition entropy decreased by ~30% from the young adult to older adult recordings (Fig. 2A; mixed-effects model: \( F_{1,18,8} = 19.17, P = 0.0003 \); paired t-test: \( t_{9} = 5.56, P < 0.0001 \)). This indicates that syllable sequencing of adult Bengalese finch song became more stereotyped over time.

To understand the nature of changes to syllable sequencing that caused the decrease in transition entropy, we examined specific changes to transition probabilities at branch points. Transition entropy is affected by the number of distinct transitions as well as the degree of skew in transition probabilities. As such, decreases in transition entropy could be driven by decreases in the number of possible transitions over time as well as increases in the degree of “dominance” of individual transitions. Such changes would be consistent with Hebbian models of synaptic and behavioral plasticity (Abbott and Nelson 2000; Dan and Poo 2004; Hebb 1949).

We first examined the degree to which the number of distinct transitions at branch point sequences changed over time. Overall, significantly fewer transitions were produced at branch points in the songs of older adults than younger adults (mixed-effects model: \( F_{1,18,8} = 9.4, P = 0.0063 \); paired t-test: \( t_{9} = 6.67, P < 0.0001 \)). This indicates that transitions were pruned from individual branch points. Indeed, 66% of branch point sequences lost at least one transition. Most of the transitions that were lost were produced infrequently when birds were young adults (n = 89 transitions; median transition probability = 6.9%; Fig. 2B). For example, 61% of the transitions that were pruned as the birds aged had transition probabilities <10% when birds were young adults. A significantly smaller proportion of retained transitions had transition probabilities <10% as young adults (38%; Fig. 2C; likelihood ratio test, \( \chi^{2} = 13.8, P = 0.0002 \)). Therefore, individual
transitions were often pruned from young adult to older adult song, and these lost transitions tended to be infrequently produced when birds were young adults.

The pruning of transitions was not due to the pruning of syllables from the male’s repertoire. As mentioned above, syllables were rarely lost over time. Furthermore, the loss of a syllable from the male’s repertoire caused the loss of a transition (up or down) as independent factors, both transition pruning and increases in the transition probabilities of dominant transitions independently contributed to a decrease in transition entropy with age. In a 2-way ANOVA with the presence of a pruned transition (yes or no) and the direction of change of the dominant transition (up or down) as independent factors, both transition pruning and increases in the transition probabilities of dominant transitions independently contributed to a decrease in transition entropy over time.

Aside from decreases in the number of transitions, increases in the prevalence of dominant (i.e., the most prevalent) transitions could also lead to decreases in transition entropy. In its most extreme form, the dominant transition could become the sole transition, thereby eliminating sequence variability. There were 14 branch point sequences that became completely stereotyped over time, and in 12 of these 14 cases it was the dominant transition that became the sole transition (sign test: $P = 0.0129$). This finding is consistent with the notion that increases in the skew of transition probabilities contribute to the age-dependent change in sequence variability.
We next broadened the analysis to all branch point sequences, not just those wherein sequence variability was lost, and assessed the consistency and importance of changes in the transition probability of dominant transitions. Transition probabilities of dominant transitions did not consistently increase over time. In only 48% of the cases did the dominant transition increase over time, which was not significantly different from 50% (sign test: $P = 0.7620$). However, increases in the transition probabilities of dominant transitions did contribute to the decrease in transition entropy. Transition entropy decreased significantly more for branch point sequences in which the dominant transition increased in prevalence than for branch point sequences in which the dominant transition decreased in prevalence over time (Fig. 2E; $t$-test: $t_{97} = 4.32, P < 0.0001$). Indeed, the decrease in transition entropy over time was significant for branch point sequences in which the dominant transition increased in prevalence ($t$-test: $H_0$: mean $= 0$; $t_{66} = 9.64, P < 0.0001$) but not for branch point sequences in which the dominant transition decreased in prevalence ($t$-test: $H_0$: mean $= 0$; $t_{50} = 1.14, P = 0.2617$).

The pruning of transitions and increases in the transition probabilities of dominant transitions affected transition entropy in similar ways. To test whether transition pruning and increases in the prevalence of dominant transitions independently contributed to the change in transition entropy, we analyzed the association between transition pruning and increases in dominance. We found that transition pruning and increases in the transition probabilities of dominant transitions were not significantly associated with each other (likelihood ratio test: $\chi^2 = 0.61, P = 0.4338$); for instance, branch points with at least one pruned transition were equally likely to have dominant transitions that increased or decreased with time. Moreover, a two-way ANOVA with the presence of a pruned transition (yes or no) and the direction of change of the dominant transition (up or down) as independent factors confirmed that both transition pruning ($F_{1,94} = 19.8, P < 0.0001$) and increases in dominant transitions ($F_{1,94} = 18.5, P < 0.0001$) independently and additively contributed to the decrease in transition entropy. Branch points with both pruned transitions and increases in the transition probability of dominants had the largest decreases in transition entropy; branch points with either pruned transitions or increases in the prevalence dominant transitions had intermediate decreases in entropy; and branch point sequences with no pruned transitions and decreases in the transition probability of dominant transitions tended to show increases in transition entropy (Fig. 2F).

Taken together, these analyses suggest that syllable sequencing becomes more stereotyped over time and that the loss of infrequently produced transitions and, to a lesser extent, increases the dominance of dominant transitions independently contribute to the age-dependent increase in stereotypy.

**Vocal motor changes across longer timescales: syllable timing.** Song tempo becomes faster and more stereotyped over time in juvenile as well as adult zebra finches (Brainard and Doupe 2001; Glaze and Troyer 2013; Kao and Brainard 2006; Kojima and Doupe 2011). Consistent with these reports, we found that adult Bengalese finch song becomes faster and more stereotyped over time. For instance, in the example depicted in Fig. 3A, the duration of the sequence “cddee” decreased from 493 ms when the male was 5 mo old to 453 ms when he was 25 mo old. Generally speaking, the durations of frequently

![Fig. 3. Age-dependent increase in song tempo and the stereotypy of song tempo. A: spectrogram (top) and mean rectified amplitude waveform (bottom) of the sequence “cddee” from a Bengalese finch. We measured sequence duration of “cddee” from the onset of “c” to the onset of the second “e”. The waveform of the sequence produced as an older adult (dashed black line) was significantly shifted to the left relative to the waveform of the bird as a younger adult (solid gray line), indicating that sequence durations were shorter when the bird was older. The dashed horizontal line indicates the thresholds used to compute sequence duration, and the bars (bottom) indicate the mean sequence durations for this sequence at both ages. B: percent changes in sequence duration (first rendition of the sequence within a song bout) for each bird as birds aged. The average change was negative and significantly different from 0, indicating that sequence durations decreased and song tempo increased. Solid line and shaded box represent mean change ± SE. C: temporal variability with which sequences were produced [i.e., interquartile range (IQR) of sequence duration for the first rendition] significantly decreased with age ($P < 0.05$). D: decreases in sequence duration were driven by decreases in the duration of intersyllable gaps. Potted are the percent changes in mean gap duration over time. The average change was negative and significantly different from 0. The duration of syllables did not change over time. Solid line and shaded box represent mean change ± SE. E: variability of gap durations also decreased with age ($P < 0.05$).](image-url)
produced sequences decreased by 3.4% from the young adult recordings to older adult recordings (paired t-test: $t_{20} = 3.03$, $P = 0.0064$). Furthermore, the variability of sequence durations (IQR) was lower for the songs of older adults than for those of younger adults (paired t-test: $t_{20} = 1.78$, $P = 0.0904$).

We repeated the analysis of age-dependent tempo changes using just the first instance of the sequence within a song because song tempo has been found to decrease over the course of a song bout (Chi and Margoliash 2001; Cooper and Goller 2006; Glaze and Troyer 2006). Furthermore, because the duration of song bouts decreased with age (paired t-test: $t_{20} = 3.33$, $P = 0.0032$), age-dependent changes to song tempo could be driven by age-dependent changes in song duration. Consistent with the previous analysis, we found significant decreases in sequence durations (paired t-test: $t_{20} = 3.08$, $P = 0.0057$) and in the variability of sequence durations (paired t-test: IQR: $t_{20} = 2.11$, $P = 0.0474$) even when we restricted our analysis to the first sequence renditions within a song bout (Fig. 3, B and C).

Changes to sequence durations could be caused by changes to the durations of syllables, gaps, or both. To analyze the contribution of gaps and syllables to age-dependent tempo changes, we computed the duration of each gap and syllable of the first instance of the focal sequence within a song bout for young and older adults. Thereafter, we computed the mean duration for each of the gaps and each of the syllables within the sequence and then summed the mean durations for all gaps and syllables of the sequence, respectively, as our measures of gap durations and syllable durations for the sequence. We found that the duration of gaps but not syllables significantly decreased over time (Fig. 3D; paired t-test: $t_{20} = 2.86$, $P = 0.0093$). We also computed the IQR of the durations of each gap and each syllable in the focal sequence, and averaged the IQRs across all gaps and syllables in the sequence as our measures of variability for gap durations and syllable durations, respectively. We found that the variability of gap but not syllable durations significantly decreased over time (Fig. 3E; paired t-test: $t_{20} = 2.26$, $P = 0.0349$). These data underscore the importance of silent gaps in the changes in song timing and support previous findings that gaps are more pliable than syllables (Cooper and Goller 2006; Glaze and Troyer 2006, 2013).

Because variance scales with measures of central tendencies, decreases in the IQR of sequence and gap durations could be driven by decreases in the median durations of sequence and gap. To test this, we used a repeated-measures analysis of covariance (RM-ANCOVA) with the median duration as a covariate. [The median is used here because the IQR is a measure of quantiles around the median and because there were significant age-dependent decreases in the median of sequence durations (paired t-test: $t_{20} = 2.99$, $P = 0.0070$) and gap durations (paired t-test: $t_{20} = 3.02$, $P = 0.0064$)]. Median durations were significantly related to the IQR of durations ($H_0$: slope = 0; $P < 0.015$ for sequences and gaps), and, moreover, controlling for age-dependent changes in median durations, the IQR of sequence and gap durations continued to be lower for older adults than for younger adults (sequence: $F_{1,20} = 3.29$, $P = 0.0839$; gaps: $F_{1,20} = 3.66$, $P = 0.0695$). This result is consistent with the finding that the magnitude of changes in the IQR of sequence and gap durations (10.6% and 8.6%, respectively) exceeds the magnitude of changes in the median duration of sequences and gaps (2.8% and 5.4%, respectively). This additional analysis supports the notion that syllable timing becomes more consistent as birds age.

**Vocal motor changes across longer timescales: syllable structure.** Age-dependent decreases in the variability of syllable structure are a hallmark of juvenile song development (Brainard and Doupe 2000; Kojima and Doupe 2011; Ölveczky et al. 2005, 2011; Ravbar et al. 2012; reviewed in Sakata and Vehrencamp 2012; Tchernichovski et al. 2001). For example, syllable variability has been found to decrease by ~50% over 1–2 mo in developing juvenile zebra finches (Ölveczky et al. 2005). Similar age-dependent changes to syllable structure have been noted in young adult zebra finches, albeit to a lesser degree (Brainard and Doupe 2001; Kao and Brainard 2006; Pytte et al. 2007). To assess age-dependent changes in adult Bengalese finches, we measured the variability of the FF of syllables with flat harmonic structure ($n = 51$ syllables; e.g., Brainard and Doupe 2001; Kao and Brainard 2006). In contrast to findings in adult zebra finches, we observed only a nonsignificant trend for decreasing variability of FF with age in adult Bengalese finches (Fig. 4; IQR: mixed-effects model: $F_{1,18.4} = 1.29$, $P = 0.2710$; paired t-test: $t_{20} = 1.72$, $P = 0.0920$). This was true regardless of whether we used the IQR or the coefficient of variation to quantify variability. The lack of significance is not likely to be due to insufficient statistical power since previous studies using fewer syllables and songs have found robust experimental effects on the variability of FF.
Temporal window of adult vocal motor changes. The boundaries of sensitive periods are determined by analyzing the degree of behavioral and neural plasticity at different ages (Hensch 2005). Understanding the degree to which the vocal motor changes we observed in the present study persist throughout life or change as birds grow older will provide insight into the temporal dynamics of vocal motor and neurobiological changes. To this end, we obtained a third song recording when Bengalese finches were even older than the previously analyzed older adult recordings for a subset of birds (n = 7). The average ages of birds at the three recording periods were 6 mo (range: 5–9 mo), 20 mo (range: 14–34 mo), and 36 mo (range: 28–55 mo), and we analyzed the magnitude of age-dependent changes in syllable sequencing at branch points and in song tempo.

For these birds, we continued to find that transition entropy changed over time (mixed-effects model: F(2,114) = 4.67, P = 0.0326; n = 24 branch points). Post hoc contrasts revealed that transition entropy was significantly lower when birds were both “older adults” (entropy: 0.7 ± 0.1, mean ± SE) and “even older adults” (0.7 ± 0.1) than when they were “young adults” (1.1 ± 0.1; Student’s t-test: P < 0.025 for both contrasts). However, sequence variability at branch points was not significantly different between “older adults” and “even older adults” (Student’s t-test: P = 0.9446). These differences in the degree of vocal motor change were not due to differences in the amount of time between recordings; the interval between the young and older adult recordings (13.6 ± 2.2 mo) was not significantly different from the interval between the older adult and even older adult recordings (16.3 ± 1.8 mo; paired t-test: t = 1.10, P = 0.3132). This suggests that the magnitude of age-dependent changes to syllable sequencing decreases over time.

To obtain a finer estimate of the temporal window of vocal motor change, we next analyzed how the magnitude of sequence changes across time varied as a function of age. In Fig. 5A, we plot the weighted change in transition entropy of individual birds at specific ages. Specifically, we computed the change in entropy across consecutive recordings (i.e., young → older and older → even older recordings) for each branch point within a bird and then computed a weighted change in entropy for each individual bird (i.e., change in entropy weighted by the prevalence of the branch point). Thereafter, we plotted the weighted change in entropy following the young adult recording (filled circles) and following the older adult recording (open circles) for each bird as a function of the branch point’s age. Consistent with our previous analyses, the weighted change in entropy from the young adult (range: 5–9 mo) to the older adult recording (range: 14–34 mo) was significantly less than zero (t-test: H0: mean = 0; t = 2.45, P = 0.0495). However, the
weighted change in entropy from the older adult recording to the even older adult recording (range: 28–55 mo) was not significantly different from zero (t-test: $t_6 = 0.31, P = 0.7697$). Furthermore, the weighted change from the young to older adult recording was significantly more negative than the weighted change from the older adult to even older adult recording (paired $t$-test: $t_6 = 2.70, P = 0.0356$). These analyses suggests that sometime between 9 and 14 mo of age the degree of age-dependent changes to syllable sequencing significantly decreases (Fig. 5A).

We repeated this set of analyses for song tempo. As per previous analyses, we focused on the first rendition of a focal sequence in a song bout to calculate sequence and gap durations. Age-dependent changes in song tempo were, overall, less robust than age-dependent changes in syllable sequencing, and as a consequence, for this smaller subset of males, we did not find a significant change in sequence or gap durations over the three time periods (RM-ANOVA: sequence duration: $F_{2,12} = 1.50, P = 0.2612$; gap durations: $F_{2,12} = 0.93, P = 0.4205$). However, age-dependent changes in song tempo generally resembled those for syllable sequencing. For example, the average proportional change in sequence duration from the young adult to the older adult recording (Fig. 5B, filled circles) was more negative than the average proportional change from the older adult to the even older adult recording (open circles; paired $t$-test: $t_6 = 0.69, P = 0.5182$). Similarly, the average proportional change in gap durations from the young adult to the older adult recording was more negative than the average proportional change from the older adult to the even older adult recording (paired $t$-test: $t_6 = 1.19, P = 0.2782$).

In addition to mean changes to song tempo, the variability of sequence and gap durations decreased as birds aged (Fig. 3). With this limited subset of males, we did not obtain significant effects of age on the IQR of sequence and gap durations (RM-ANOVA: IQR of sequence durations: $F_{2,12} = 1.14, P = 0.3534$; gap durations: $F_{2,12} = 3.19, P = 0.0776$). However, just as for mean values, the average proportional change in the IQR of sequence durations from the young adult to the older adult recording (Fig. 5C, open circles; paired $t$-test: $t_6 = 0.72 P = 0.5003$), and the average proportional change in the IQR of gap durations from the young adult to the older adult recording was more negative than the average proportional change from the older adult to the even older adult recording (paired $t$-test: $t_6 = 1.82, P = 0.1188$).

**DISCUSSION**

Vocal motor changes beyond the sensitive period for vocal plasticity. Birdsong is a vocal motor behavior that, like speech, is learned during a sensitive period in development. During the “sensorimotor phase,” juvenile songbirds “babble” and engage in vocal motor practice, which leads to rapid increases in vocal consistency across development (Brainard and Doupe 2000, 2001; Catchpole and Slater 2008; reviewed in Doupe and Kuhl 1999; Mooney 2009; Tchernichovski et al. 2001). Whereas juvenile song is widely considered plastic, adult song has historically been considered as “crystallized” because the gross structure of song remains stable following sexual maturation (3–4 mo) and because neural plasticity mechanisms are progressively lost over development (Brainard and Doupe 2013; Mooney 2009; Sakata and Vehrencamp 2012). However, plasticity mechanisms are not absent in adult songbirds, and adult vocal performance could continue to change (e.g., become more stereotyped) over time. Consistent with this notion, we found that the sequencing and timing of song syllables naturally became more stereotyped as young adult Bengalese finches (~6 mo) aged into older adults.

The manner in which song elements (syllables) are sequenced became more stereotyped with age (Figs. 1 and 2). This increase in sequence stereotypy was caused by a reduction in the number of distinct transitions produced at branch point sequences as well as an increase in the skew and predictability of transition probabilities. In the majority of branch point sequences, at least one transition was “pruned,” and in a handful of cases (14%), all but one of the transitions were pruned such that sequences became completely stereotyped. Furthermore, for half of the branch point sequences, the most frequently produced transition of the branch point (i.e., the dominant transitions) became more prevalent, which also contributed to age-dependent increases in sequence stereotypy for those cases. As a consequence, our data indicate the presence of neural mechanisms that drive changes to syllable sequencing over months in adult Bengalese finches.

The evidence for neural mechanisms that drive changes to syllable sequencing seems to contrast with a previous study revealing active mechanisms to maintain transition probabilities in adult Bengalese finches. Using a targeted reinforcement paradigm, Warren et al. (2012) experimentally drove changes to transition probabilities and then observed that transitions probabilities returned to baseline levels within days following the termination of reinforcement. Consequently, the authors concluded that there exist brain mechanisms that rapidly and actively maintain transition probabilities in adult Bengalese finches. Although the ages of birds between our study and that of Warren et al. (2012) seem comparable, a noticeable difference between the studies is the timescale of observed vocal motor changes: we observed changes to transition probabilities over months to years, whereas Warren et al. (2012) observed the reestablishment of transition probabilities over days. Therefore, it is possible that there exist distinct mechanisms that act on shorter and longer timescales: one mechanism could act on shorter timescales to maintain transition probabilities, whereas another mechanism could act on longer timescales to slowly drive changes to transition probabilities.

Despite the presence of mechanisms to increase sequence stereotypy, in most cases sequence variability was not completely eliminated. Indeed, sequence variability was maintained in 86% of the branch points analyzed and was present even in our oldest birds (>5 yr). This suggests that there also could exist mechanisms to actively maintain sequence variability. Results from a previous study (Warren et al. 2012) suggest a similar phenomenon because sequence variability returned after experimentally driving increases in sequence stereotypy. Such a mechanism could be functionally significant since studies in the Bengalese finch and related species suggest that sequence variability is ethologically important (reviewed in Okanoya 2012). Taken together, when our observations are combined with those of Warren et al. (2012), the data suggest the existence of multiple mechanisms in the brain that act at different timescales and in different directions.
In addition to changes to syllable sequencing, we observed age-dependent changes to the timing of syllable sequences. In particular, we found that song tempo and the stereotypy of song tempo increased with age (Fig. 3). These changes were due to a decrease in the mean and variability of intersyllable gap durations but not to syllable durations. Such changes are analogous to developmental changes in syllable timing and suggest a shared mechanism underlying temporal changes in juvenile and adult songbirds (Glaze and Troyer 2013). For example, because intersyllable gaps coincide with short inspirations during song (Aronov et al. 2011; reviewed in Goller and Cooper 2004; Veit et al. 2011), the shortening of gaps could indicate that birds are more efficient at replenishing their air supply with shorter inspirations.

The robust changes to the temporal structure of adult Bengalese finch song contrast with the lack of significant changes to the spectral structure of song with age. Whereas the structure of song syllables (e.g., fundamental frequency) becomes more consistent over weeks during juvenile development (e.g., Okanoya 1997; Tchernichovski et al. 2001), we did not observe significant changes to the stereotypy of spectral features over months in adult Bengalese finches. This lack of age-dependent increases in syllable consistency contrasts with observations of age-dependent increases in syllable consistency in adult zebra finches (Kao and Brainard 2006; Pytte et al. 2007). One potential explanation for this difference is that previous studies examined age-dependent changes in younger adult songbirds. For example, Kao and Brainard (2006) observed changes in the stereotypy of fundamental frequency in 3-mo-old zebra finches, whereas the average age of our young adults was 6 mo (range: 4–11 mo). The difference in findings could also reflect a species difference in the nature of adult vocal motor change. Regardless of the underlying cause, our data indicate that the spectral structure of adult Bengalese finch song changes less over time than the temporal structure of song. This finding is consistent with models of song learning suggesting that birds first master the structure of syllables and then master the sequencing of syllables (e.g., Tchernichovski et al. 2000; Troyer and Doupe 2000a, 2000b).

A finer grained analysis of the temporal window of adult vocal motor change suggests that the magnitude of age-dependent changes to syllable sequencing and timing decrease after 1 yr of age (Fig. 5). Specifically, we found that the degree to which syllable sequencing and timing changed over time was reduced when birds were >14 mo old relative to when they were 5–9 mo old. This was especially conspicuous for syllable sequencing (Fig. 5A). These data suggest that the temporal organization of song stabilizes after 1 yr of age. However, these analyses do not rule out the possibility that birds >1 yr of age retain the capacity for change but that the rate of change is dramatically reduced after 1 yr. Nevertheless, this analysis highlights the dynamic nature of vocal motor change in adult songbirds and suggests age-dependent changes in the neural control of song in adult songbirds.

Potential mechanisms of vocal motor change. Birdsong is controlled by discrete and specialized neural circuits (Brainard and Doupe 2013; Mooney 2009). For example, the sequencing and timing of syllables is regulated by the intrinsic and recurrent circuitry within and between HVC and RA (robust nucleus of the arcopallium; Brainard and Doupe 2013; Jin 2009; Mooney 2009; Mooney and Prather 2005; Prather 2013). Neural activity in HVC encodes syllable sequencing (Fujimoto et al. 2011; Hahnloser et al. 2002; Schmidt 2003; Vu et al. 1994), and locally cooling HVC slows the intrinsic dynamics of HVC activity and decreases song tempo (Andalman et al. 2011; Long and Fee 2008; Long et al. 2010). Perturbations of activity in HVC and RA lead to significant changes to syllable sequencing and timing (Ashmore et al. 2005; Vu et al. 1994; Wang et al. 2008). As such, variability in the sequencing and timing of syllables are likely to be caused by variability in the patterns of activity in HVC and RA. Moreover, age-dependent changes in the stereotypy of syllable sequencing and timing are likely to be driven by age-dependent changes in the patterns of neural activity in HVC and RA.

The anterior forebrain pathway (AFP) is a forebrain-basal ganglia-thalamic loop that is critical for various forms of song plasticity (reviewed in Brainard and Doupe 2013). We do not, however, hypothesize that the AFP plays a major role in the age-dependent changes observed here. Whereas the AFP has repeatedly been found to regulate the control and plasticity of syllable structure (Andalman and Fee 2009; Charlesworth et al. 2012; Kao and Brainard 2006; Kao et al. 2005; Warren et al. 2011), the AFP seems to play a lesser role in the control and plasticity of syllable sequencing and timing (e.g., Ali et al. 2013; Hampton et al. 2009; Stepans and Doupe 2010). For example, lesions of LMAN, the output of the AFP, affect the variability of syllable structure but not the variability of syllable sequencing or song tempo in adult Bengalese finches (Hampton et al. 2009). Lesions of LMAN and the basal ganglia nucleus Area X prevent feedback-driven plasticity in syllable structure but not in the timing of syllables (Ali et al. 2013). Although there exists some evidence that the AFP can influence syllable sequencing and timing (e.g., Brainard and Doupe 2001; Goldberg and Fee 2011; Hamaguchi and Mooney 2012; Olvecky et al. 2005; Scharff and Nottebohm 1991; Thompson et al. 2011), we argue that the AFP is not the primary locus of adult vocal change in Bengalese finches because studies documenting AFP influences on syllable sequencing and timing also observe AFP influences on syllable structure. For example, infusions of bicuculline into LMAN increase the variability of syllable sequencing as well as the spectral entropy of individual song syllables in adult zebra finches (Hamaguchi and Mooney 2012), and inactivations of LMAN decrease sequence variability as well as the variability of syllable structure in juvenile zebra finches (Olvecky et al. 2005). Because we did not observe significant changes to the variability of syllable structure in adult Bengalese finches, our findings are inconsistent with the model that the AFP drives age-dependent changes to Bengalese finch song.

Age-dependent vocal motor changes could also be caused by changes in circuits outside HVC, RA, and the AFP. For example, it has been hypothesized that circuitry involving the shell region of LMAN and dorsal arcopallium (Ad) could contribute to the plasticity of syllable sequencing (Bottjer and Altenau 2010). Subsequent studies should investigate how activity in the shell region of LMAN and Ad influences syllable sequencing and timing in the Bengalese finch.

Regardless of the loci of neural changes, changes to syllable sequencing and timing suggest the possibility that plasticity mechanisms are engaged during song production in adult
Bengalese finches. Indeed, the increases in the stereotypy of syllable sequencing and timing are consistent with the notion that adult song, like juvenile song, represents a form of vocal practice. Generally speaking, practice leads to increases in the consistency with which motor acts are performed (Costa 2011; Davids et al. 2006; Ericsson and Lehmann 1996; Glaze and Troyer 2013; Madison et al. 2013; Magill 2004; Müller and Sternad 2009; Vihman 1996). For example, with practice, singers become more adept at “hitting” desired notes, drummers improve their ability to produce beats at a consistent tempo, and piano players make fewer errors in the sequencing and timing of notes (e.g., Ericsson et al. 1993; Konczak et al. 2009, Sakai et al. 2004). Experimental studies in adult songbirds indicate that manipulations of auditory feedback can activate plasticity mechanisms to drive vocal motor change but relatively little is known about degree to which adult birdsong naturally changes over time in a manner consistent with the practice framework (reviewed in Brainard and Doupe 2013; Sakata and Vehrencamp 2012). Our finding that adult Bengalese finch song becomes more stereotyped over time is consistent with this framework and supports the hypothesis that plasticity mechanisms could be naturally engaged during adult song production.

Hebbian mechanisms can explain various forms of behavioral and synaptic plasticity, including vocal motor plasticity in songbirds (Abbott and Nelson 2000; Dan and Poo 2004; Hanuschkin et al. 2013; Hebb 1949; Troyer and Doupe 2000a, 2000b). Models of song production hypothesize that syllable sequencing is regulated by functional connections between populations of neurons in vocal premotor regions (e.g., HVC) that encode song syllables (reviewed in Jin 2009; Katabira et al. 2011; Nishikawa and Okanoya 2006). Furthermore, these models propose that differences in transition probabilities at branch point sequences reflect differences in the strengths of functional connectivity between populations of neurons; infrequently produced transitions reflect weak functional connections and dominant transitions represent strong functional connections. Hebbian processes could lead to a weakening of weak connections and the strengthening of strong connections between populations of neurons encoding syllables. In an extreme form, such plasticity could resemble “winner-take-all” models of synaptic development in which a single connection remains after the pruning of weaker synapses (Abbott and Nelson 2000; Troyer and Doupe 2000a, 2000b). In our data, both the pruning of infrequently produced transitions and the examples in which the transition probability of dominant transitions increase over time are consistent with Hebbian models of plasticity. Furthermore, branch point sequences that became completely stereotyped over time are intriguing examples of a winner-take-all phenomenon because in most of these cases the dominant transition became the sole transition. However, in contrast to the predictions of a Hebbian framework, we did not observe an overall trend for dominant transitions to become more prevalent over time. Indeed, dominant transitions were equally likely to increase or decrease in prevalence, and in some instances, previously nondominant transitions became dominant over time (e.g., Fig. 1D). The pruning of infrequent transitions and changes to dominant transitions were largely independent, suggesting that distinct mechanisms could regulate distinct types of age-dependent changes to syllable sequencing.

Age-dependent changes to syllable timing are also consistent with a Hebbian model of adult song plasticity. With repeated singing, Hebbian mechanisms could lead to increases in the strengths of the functional connections between populations that encode adjacent song syllables (Jun and Jin 2007; Nishikawa and Okanoya 2006). As functional connections between populations of neurons that encode adjacent syllables become stronger, the interval between syllables should decrease. We observed such decreases to intersyllable gaps (Fig. 3), suggesting that Hebbian mechanisms could also underlie age-dependent changes in the timing of song syllables. Independent of Hebbian processes, age-dependent changes in syllable sequencing and timing could also reflect age-dependent changes in the sensory control of song. Both the sequencing and timing of adult song are critically dependent on auditory feedback (reviewed in Brainard and Doupe 2000; Mooney 2009; Prather 2013; Tschida and Mooney 2012). In the Bengalese finch, deafening leads to changes in syllable sequencing within days, and acute perturbations of auditory feedback lead to real-time changes in the sequencing and timing of syllables (Okanoya and Yamaguchi 1997; Sakata and Brainard, 2006; Woolley and Rubel 1997). The rapidity with which deafening alters song has been found to decrease with age in a related songbird species, the zebra finch, suggesting that adult song becomes less dependent on auditory feedback as adult birds continue to master the performance of song (Brainard and Doupe 2001; Lombardino and Nottebohm 2000). As a consequence, age-dependent increases in sequence stereotypy and song tempo could similarly reflect a decreased dependence on auditory feedback in Bengalese finches. Consistent with this notion is the finding that perturbations of auditory feedback cause smaller changes to song when Bengalese finches produce the more stereotyped and faster courtship song than when they produce the more variable and slower non-courtship song (Sakata and Brainard 2009). Because syllable sequencing and timing tend to be more stable in adult Bengalese finches >1 yr of age, it would be interesting to assess whether perturbations of auditory feedback have more rapid and dramatic effects on the temporal organization of song in Bengalese finches <1 yr of age than in birds >1 yr old.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.S.J. and J.T.S. performed experiments; L.S.J. and J.T.S. analyzed data; L.S.J. and J.T.S. interpreted results of experiments; L.S.J. and J.T.S. prepared figures; L.S.J. and J.T.S. drafted manuscript; L.S.J. and J.T.S. edited and
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