Diurnal oscillation of vocal development associated with clustered singing by juvenile songbirds

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Abstract

Spaced practice affects learning efficiency in humans and other animals. However, it is not well understood how spaced practice contributes to learning during development. Here we show the behavioral significance of singing frequency in song development in a songbird, the zebra finch. Songbirds learn a complex song pattern by trial-and-error vocalizations as self-motivated practice, which is executed over a thousand times per day during the sensitive period of vocal learning. Notably, juveniles generate songs with a high frequency of singing in clusters with dense singing, whereas adults sing in the opposite way. This juvenile-specific clustered singing was characterized by clear separations of daily time for intent practice and rest. During the epochs of vocal practice in juveniles, the song structure approached that of song produced at the end of the day. In contrast, during the epochs of vocal rest, the structure of juvenile songs regressed toward that of songs produced at the beginning of the day, indicating a dynamic progression and regression of song development over the course of day. When the singing frequency was manipulated to decrease at the juvenile stage, the oscillation rate of song development was dramatically reduced. Although the juvenile-specific clustered singing was driven in nontutored socially isolated or early-deafened birds, the diurnal oscillation of vocal development was only observed in nontutored isolated juveniles. These results show the impact of “self-motivated” vocal practice on diurnal song developmental plasticity, modulated by the amount of vocal output and auditory feedback.
Introduction

Memory and learning are a consequence of multiple experiences. The frequency and timing of training experiences are crucial for learning efficiency. Experiences distributed over time (spaced training) are more easily encoded than a single prolonged experience (massed training) (Dudai and Eisenberg, 2004; Ebbinghaus, 1885; Tully et al., 1994). Further, neural activity associated with spaced or massed training leads to different levels and types of gene expression via different intensities of signal cascade activities (Naqib et al., 2012; Pagani et al., 2009). Although most studies have been performed under well-controlled conditions regulated with trial numbers, frequency, and duration, it is not well understood how “voluntary action-based (self-motivated)” spaced practice contributes to learning during development.

The zebra finch (*Taeniopygia guttata*), a songbird, provides a unique model system for observing the behavioral impacts of voluntary action on learning a complex motor pattern: self-motivated singing practice for song learning. Male zebra finches develop their songs between 25 and 120 days post-hatch (dph), a critical/sensitive period for vocal learning (Immelmann, 1969; Zann, 1996). This period includes two phases, the sensory- and sensorimotor-learning phases. In the sensory-learning phase (Fig. 1A), juveniles acquire sensory memories of songs by hearing matured birds’ songs as a template to imitate. The sensorimotor-learning phase starts from approximately 30 dph in the context of soft, highly variable, and discriminable sounds of subsongs (Fig. 1A). Thereafter, produced song, called plastic song, gradually includes recognizable yet variable syllables without a fixed temporal sequence order. In the early plastic song phase, syllable acoustic structures, such as entropy variance (EV) and duration, are changed in a single day (Deregnaucourt et al., 2005). Both syllable acoustic features and their sequence order then become more stable (Tchernichovski et al., 2001). After 90–120 dph, the zebra finch produces its song as a crystallized song. Through song development, songbirds produce over hundreds of songs every day as
self-motivated singing. This singing is not produced as a response to or aimed toward a specific individual but it is generated by undirected practice. The total number of song bouts produced during the critical period of vocal learning correlates with the number of learned song syllables and stability of the syllable sequence (Johnson et al., 2002). This correlation suggests the importance of an appropriate singing amount at the critical period for the acquisition of a qualified song. However, the regulation and importance of the timing and frequency of spontaneously produced vocalizations remains poorly understood.

Here we investigated phenotypic changes of diurnal frequency and timing of singing during song development in the zebra finch. Juveniles generate songs with a high frequency of singing in sporadic clusters, whereas adults produce a low frequency of singing in short singing clusters. To examine the behavioral significance of clustered dense singing by juveniles in song development, we performed a population analysis of acoustically modified syllables. We found a diurnal oscillation of song development, which was interfered by disturbance of dense singing or deficit of auditory feedback at the juvenile stage.
Results

Developmental change in the diurnal distribution and frequency of singing

To elucidate the developmental dynamics of song production through the critical period of vocal learning, we first compared the distribution and frequency of diurnal singing between two developmental stages (50–55 dph as an early plastic song phase and 100–105 dph as a crystallized song phase). The numbers of song bouts per 5-min bins over two consecutive days were calculated for both stages and are represented as singing histograms (Fig. 1B, blue) and heat maps (Fig. 1B, gray to red). The total number of diurnal singing bouts in the juvenile stage was almost the same or slightly higher than that in the adult stage, as previously reported (Johnson et al., 2002). In contrast, the diurnal distribution and frequency of singing showed differences between the two stages (Fig. 1B). Juveniles at approximately 55 dph sang sporadically with an increased frequency followed by long resting intervals. Adults sang continuously during the day with a low frequency and short resting intervals. The distinct song productions between the two stages encouraged us to further investigate the long-term developmental changes of diurnal distribution and singing frequency during the critical period of vocal learning.

We accordingly recorded the entire vocal activity from 28 to 40 dph when juveniles began to sing until their songs were crystallized at 105–110 dph (n = 4). We then analyzed the distribution of song production, bout numbers, and daily frequency of singing per 5-min bins throughout the song developmental period. Sonograms and heat maps of singing frequency depicted the developmental changes in diurnal song production through the critical period (Fig. 2A and B as an example). High-frequency singing (Fig. 2B, red) was observed at approximately 50–75 dph, when the bird sang plastic songs. Low-frequency singing (Fig. 2B, yellow to gray) was observed from 80 dph, when the birds started singing stable song patterns in some song renditions. The total numbers of song bouts in a day began to increase at approximately 45–50 dph, peaked at 60–75 dph, and then gradually decreased in the adult stage (Fig. 2C). Two behavioral indices, “singing cluster” and “singing density,”
were calculated with the aim of quantifying the developmental phenotypic transition of singing activity (see Materials and methods). We defined a “singing cluster” as a continuous session of singing that was preceded by more than 5 min of silence (no singing) and a “singing density” as the average number of song bouts in singing clusters. The number of singing clusters gradually increased from 70 dph to the adult stage (Fig.2D and F). In contrast, the diurnal singing density showed an inverse distribution relative to the diurnal distribution of singing clusters through development (Fig.2E and G). The singing density showed a large variation among singing clusters in a day, although the median of singing density in a day was highest in the plastic song phase at 50–65 dph after which it gradually decreased until songs were more crystallized (Fig. 2E and I). For quantitative analyses, the developmental change in song production was compared at three developmental stages: the early plastic song phase (43–57 dph), the late plastic song phase (74–87 dph), and the crystallized song phase (100–110 dph). Four singing phenotypes here singing cluster, singing bout number per singing cluster, duration of a singing cluster, and singing density (Fig. 2F–I). For any singing phenotype, the juveniles in the early plastic song phase showed significant differences from the late plastic song and crystallized song phases (n = 4 birds, Fisher’s PLSD test, P < 0.01–0.001). This indicated that the zebra finch in the early plastic song phase produced high-singing density in sporadic but long singing clusters, generating spaced time with practice and rest in a day. In contrast, no significant difference was observed between the late plastic and crystallized song phases, showing that the birds in both these phases produced continuous singing patterns with a low-singing density in the majority of short singing clusters. These results show clear differences in diurnal song production between the song-learning phase and the later learning phases.

Singing clusters associated with high-dimensional changes in daily song development

We investigated the behavioral parameters of song development that corresponded with the juvenile-specific singing phenotypes. Syllable acoustic features, such as EV, are known to
change in a single day in the juvenile stage (Deregnaucourt et al., 2005; Deshpande et al., 2014). Accordingly, we first calculated the diurnal transitions in the EV of syllables at the beginning and end time points of each singing cluster and a time point 3–4 h after lights turned on. As previously reported (Deregnaucourt et al., 2005), the EV of some but not all syllables dramatically changed only within a few hours after the onset of diurnal singing (Fig. 3A and B), indicating that the rate of change of syllable EV was independently regulated from the timing and duration of singing clusters (Fig. 3B).

We then evaluated the relationship between the production of singing clusters and a higher-dimensional change of song development. We used information from two acoustic parameters and population rate of produced syllables, given that the juveniles must change multiple parameters of song features and not only one syllable acoustic parameter during song development (Fig. 3A). For this purpose, syllable populations at selected time points in a day were visualized as density plots separated by syllable duration and mean frequency modulation (FM) and compared with the population at other time points (Fig. 3B, bottom). There were apparent differences between distributions of the earliest and latest syllable populations within singing clusters (Fig. 3B, bottom). The syllable population at an early time point of each singing cluster exhibited a subtle but consistently different distribution than one at a late time point in the same singing cluster, indicating a population change of acoustically modified syllables during singing clusters. We analyzed transitions of the syllable populations by the calculation of the Kullback–Leibler (K–L) distance, allowing the quantification of acoustic changes of syllables and their occurrence through a day (Wu et al., 2008). The K–L distance at each time point was calculated from the earliest syllable population (Fig. 3B, orange) or the latest syllable population in a day (Fig. 3B, red). In comparison with the earliest syllable population, a divergence of syllable density population was induced during singing clusters and a backset of transition occurred during resting times between singing clusters (Fig. 3B, orange). Symmetrical diurnal dynamics of the syllable population was observed in comparison with the latest syllable population of the day (Fig.
The diurnal dynamics of the syllable populations was quantitatively verified in the early plastic song phase (45–66 dph, n = 9; Fig. 3C). The rate of change of the K–L distance in a day was calculated at five time points: at three singing clusters (the first, middle, and last clusters in a day) and at two resting interval times preceding the first and middle clusters. The midmost singing cluster of the day was used as the middle cluster. During the singing clusters, the rate of change of the syllable populations showed positive values, indicating that the distribution of the syllable population shifted toward that of the latest syllable population in the day (Fig. 3C). In contrast, during the two resting intervals, the highest rate of change of the K–L distance showed a negative value, indicating that the distribution of the syllable populations during the resting intervals shifted in the opposite direction from that of the latest population in the day (Fig. 3C). Thus, among the five time points in a day, the rate of change of the syllable population significantly differed (one-factor ANOVA, P < 0.0001), indicating that singing practices and their following rest times showed oscillations of hourly progression and regression of song development as higher-dimensional parameters during a day at the juvenile stage.

**Juvenile-specific singing frequency regulates the oscillation rate of diurnal song development**

The number of diurnal singing clusters and singing density was regulated with an inverse relation through development. Therefore, juveniles generate songs with a “high” frequency in “sporadic” singing clusters at the early plastic song phase (Fig. 2F and I). To elucidate the behavioral significance of juvenile-specific clustered singing in song development, we then examined whether the diurnal change of song development would be affected by manipulation of singing density at the juvenile stage. For this, we manipulated the birds to sing with two different singing frequencies while maintaining a similar number of singing clusters and a similar number of singing bouts in 2 days (60–67 dph, n = 4; Fig. 4A as two examples). As the result, birds were maintained to produce lower singing frequency in any
clusters in the experimental day than subsequent high (normal)-singing frequency day (average singing density in the day, mean ± SEM = 2 ± 0.3 or 7 ± 0.9, respectively). Diurnal change in the syllable populations was then compared with the earliest and latest syllable populations in the two successive days. The oscillation of distribution of the syllable population in the low-singing frequency group was diminished (Fig. 4B, left). In contrast, the high-frequency singing group (Fig. 4B, right) maintained a distinct diurnal oscillation of acoustically modified syllable populations against the low-frequency singing day (repeated-measures ANOVA, $P < 0.001$). Although it would be crucial to consider a potential stress effect caused by the manipulation of singing frequency, these results suggest that the juvenile-specific dense singing contributes to the enhancement of the oscillation range of diurnal song development.

**Intrinsic regulation of clustered singing and auditory-dependent modification of diurnal oscillation of song development**

We then tried to elucidate the external and/or internal factors that contributed to the regulation of clustered singing and diurnal oscillation of song development. For this purpose, juvenile birds were placed under two conditions, nontutored social isolation or auditory deprivation. We compared their diurnal song production in two developmental stages, the juvenile (39–66 dp h) and adult (100–127 dph) stages. The two experimental procedures started before the sensory learning period for vocal learning (see Materials and methods). The nontutored, solitary-isolation birds exhibited a tendency for generating “parts” of a motif structure with prolonged and variable syllables. However, the stabilizing time point of acoustic features for majority of the syllables was similar to that in the normal birds. In contrast, the early-deafened birds continued generating variable song structures with fewer harmonic syllables even at the adult stage (100–150 dph), although they finally exhibited stabilized song structures characterized by the stable temporal sequences of distinguishable syllables by approximately 300 dph (Mori and Wada, 2015). Under both reared conditions,
the birds still showed significant differences between the juvenile and adult stages in the number of singing clusters and/or singing density (Fig. 5A, paired *t*-test, *P* < 0.05–0.001). In a singing cluster, both socially isolated and early-deafened birds represented an increasing trend through development as observed in live-tutored birds (Fig. 2F). Conversely, singing density decreased through the development, even in socially isolated and early-deafened birds, as similarly represented in live-tutored birds (Fig. 2I). Further, the socially isolated and early-deafened birds generated a similar number of singing clusters and density as those produced by normal live-tutored birds. This result suggests the existence of an innate ability to regulate the timing and frequency of diurnal vocal production through individual development. We then analyzed the oscillation rate of diurnal song development at the juvenile stage of the two conditions. Although juveniles generated songs without a tutor song under both nontutoring social isolation and auditory deprived conditions, the diurnal oscillation of vocal development was observed in socially isolated juveniles but not in deafened ones (Fig. 5B, one-factor ANOVA, *P* < 0.0001). These results indicate an auditory feedback-dependent modification of diurnal oscillation of song development. Furthermore, by calculation of the K–L distance between the earliest and latest songs in a single day, we found a significant difference of diurnal song development rate between normal intact and early-deafened birds (Fig. 5C, Scheffé's F test, *P* < 0.01).
Discussion

To elucidate the development of singing phenotypes and its behavioral significance in song learning, we investigated the developmental dynamics of diurnal singing during the critical period of vocal learning. We found that the zebra finch shows phenotypic changes of diurnal frequency and timing of singing throughout song development. Notably, juveniles start singing with an increased frequency in compact singing clusters with singing practices and rests in a day. The juvenile-specific clustered singing practices and rests generated an hourly progression and regression of song development, as shown by the oscillation of high-dimensional modification of syllable acoustics during the day. Furthermore, when the singing frequency was manipulated to decrease, while maintaining rendition clusters and total singing amount, the oscillation range of diurnal song development dramatically reduced. In a previous study, during the rapid song learning paradigm in the early plastic song phase, a pronounced deterioration in song structure was observed after night sleep and even after a melatonin-induced daytime nap (Deregnaucourt et al., 2005). Although we speculated that the observed diurnal oscillation of syllable acoustics had been affected by daytime naps during the singing rests, the juveniles in this study remained in a waking state and exhibited regular eating, drinking, and jumping in their cages. Thus, our study describes the sleep-independent song oscillation process during the daytime. Furthermore, the same previous study revealed that daily improvement occurred during morning singing, but little improvement occurred thereafter in a day in the rapid song-learning paradigm. In contrast, we observed a continuous oscillation of song development through the daytime, with a larger oscillation in the morning (Fig. 3C). This difference between studies may be associated with the use of different behavioral parameters to characterize song development such as an acoustic feature (EV), and similarity score to the song model in the previous study versus the K–L distance between the two-dimensional occurrence rates of two acoustic features in this study. Although we observed that the EV of a set of some but not all syllables changed within a few hours after the onset of diurnal singing (Fig. 3B), it would be highly conceivable that a different acoustics parameter changes at a different time course at
a different set of syllables, causing the independent regulation of multiple acoustic features for each syllable. Although it will be necessary to consider differences in the biological meaning of the investigated behavioral parameters between studies in the future, the accumulated studies suggest the existence of an hourly and daily-based song developmental oscillation in the juvenile learning stage.

Training distributed over time, spaced training, is generally considered more effective for memory and learning formation than training presented with little or no rest interval, massed training. This phenomenon is known as the spacing effect and was first described by Ebbinghaus (1885). This spacing effect is widely recognized in many learning tasks such as operant conditioning, spatial learning, verbal and nonverbal recall tasks, and motor learning in many species (Menzel et al., 2001; Okamoto et al., 2011; Pagani et al., 2009; Vlach et al., 2008; Wahlheim et al., 2011). However, most of the past studies were based on the observation of well-controlled enforced tasks for which trial numbers and durations were strictly regulated by the experimenters. Furthermore, the developmental influence of spacing effects has not yet been well studied. The zebra finch would be an excellent animal model for addressing the behavioral and developmental impacts of spaced training that is voluntarily generated during developmental learning. In this study, we found that manipulation of singing inhibition, social isolation, or deafening does not affect the development of clustered singing, indicating an intrinsic regulation of singing frequency and timing through the critical period of vocal development. However, singing inhibited or deafened juveniles did not show distinct diurnal oscillation of song development. In contrast, socially isolated (nontutored) juveniles retained their oscillation of song development, as did live-tutored birds. These results suggest that the diurnal oscillation of song development is modulated by auditory feedback and daily amount of singing but tutor memorization is not necessary. Taken together, the oscillations of song development may contribute to repeated opportunities to reshape previously developed motor skills. Further experiments, such as the manipulation of singing frequency and timing through entire developmental stages, are
needed to evaluate the behavioral effect of “self-motivated” clustered vocal practice on vocal development and learning efficiency.

Singing during development is not usually generated in response to or directed toward specific individuals, but rather generated as undirected and self-motivated behavior. As previously reported (Johnson et al., 2002), total amounts of diurnal singing were similarly maintained between the juvenile and adult stages (Fig. 1B and 2C). In contrast, diurnal frequency and timing of singing are differentially regulated through development in an intrinsic manner (as observed for socially isolated and deafened birds in a sound attenuation box), suggesting that neural substrates innately regulate the developmental change of singing frequency and timing in a day. Similar self-motivated and clustered dense singing has been observed in another songbird species, the Bengalese finch (Lonchura striata var. domestica), at the juvenile stage (unpublished data of our group). These findings suggest that the self-motivated vocal practice in the juvenile stage may have evolved and been maintained as an adaptive learning strategy for vocal learning. Whether the self-motivated “spaced training/practice” of vocalization is generally observed in other vocal learners such as parrots, cetaceans and humans are yet to be investigated. Multiple lines of recent studies in songbirds showed a potential link of enkephalin and mu-opioid receptors in the medial preoptic nucleus and ventral tegmental area, which are highly conserved in mammals and birds to regulate undirected singing (Khurshid et al., 2010; Ritters et al., 2005). However, the neural mechanisms underlying self-motivated singing behavior have not been well characterized, especially in the developmental changes of singing frequency and timing.

Although in this study we focused on diurnal song development associated with juvenile stage-specific singing phenotypes, it is still unclear whether or how juvenile stage-specific singing phenotypes contributes to the molecular basis of song-learning efficiency during the critical period of vocal learning. Distinct singing frequencies and timing between the juvenile and adult stages should affect the temporal dynamics of molecular expression differently in the song system, especially in singing-driven (neural activity-dependent) gene
expression. *Egr1* in the robust nucleus of the arcopallium RA and *Penk* in the premotor nucleus HVC are more highly induced by singing in juveniles than in adults even with similar total singing durations (Jin and Clayton, 1997; Wada et al., 2006; Whitney et al., 2000). However, the factors directly regulating distinct expressions for juvenile and adult stages are unknown. The molecular function of a neural activity-dependent gene is maintained by multiple regulatory steps, including regulation of intracellular calcium concentration, signaling cascades for transcriptional induction, timing of mRNA transfer/degradation, protein translation, and epigenetic states of promoter domains (Bramham, 2008; Carulli et al., 2011; Clayton, 2000; West and Greenberg, 2011). The molecular machinery of these steps is also regulated by the frequency and duration of neural activity (Bramham et al., 2008; Rudenko et al., 2013; Tang et al., 2002). Thus, juvenile-specific singing phenotypes and clustered dense singing may be a driving force for enhancing behavior-driven neural plasticity for song development and learning efficiency, associated with individual differences in the song developmental trajectory.
Materials & Methods

Definition

In this study, we define the words (i) song bout, (ii) singing cluster, and (iii) singing density as follows. (i) A “song bout” is a continuous production of songs that are separated by intervals. The intervals between song bouts are defined as time periods longer than three times the mean value of 50 inter-syllable intervals randomly selected each day. (ii) “Singing cluster” is defined as a continuous session of singing preceded and followed by more than 5 min of silence (without singing). (iii) “Singing density” is defined as the average number of song bouts in a singing cluster.

Animals

Twenty-eight male zebra finches were obtained from our breeding colony at Hokkaido University. The birds were kept in breeding cages on a 13: 11-h light : dark cycle. During song recording sessions, each bird was individually housed in a sound-attenuating box. For the analyses of singing frequency through song development, the birds were raised with their families until 34 dph and transferred with their biological father \((n = 2)\) or an unrelated adult male \((n = 2)\) into a sound-attenuating box and were then housed in the box until 110 dph for song recording. Solitary-isolation birds were transferred with their mother into a sound-attenuating box before 5 dph and kept from adult males. At 30–35 dph, the mothers were removed from the box, and the birds were housed alone. An example of song development of the solitary-isolate birds is provided in a separate study (Mori and Wada, 2015). All experiments were conducted under the guidelines and approval of the Committee on Animal Experiments of Hokkaido University. The guidelines are based on the national regulations for animal welfare in Japan (Law for the Humane Treatment and Management of Animals, after a partial amendment No.105, 2011).

Song recording and analysis

Songs were recorded with a unidirectional microphone (SM57, Sure, Chicago, USA)
connected with a computer with the sound-event triggered software, Sound Analysis Pro v1.04 (Tchernichovski et al., 2000).

To measure song developmental changes in a single day, we quantified changes in syllable acoustic features and syllable populations as two-dimensional scatter density plots. We measured the Wiener EV, mean FM, and syllable duration and amplitude. The segmentation of each syllable structure was identified based on amplitude and Wiener entropy thresholds using Sound Analysis Pro. We used (i) Wiener entropy variance for quantifying the transition of a syllable acoustic feature using 50 syllables from the beginning or the end of each singing cluster and (ii) mean FM and syllable duration for calculating the Kullback–Leibler (K–L) distance (Wu et al., 2008). The K–L distance was adapted as a way to measure the distance between two sets of syllable populations by comparing their probability density distributions. We generated scatter density plots of syllable populations by two acoustic features: syllable duration (ms; denoted as \( m \)) and mean FM (denoted as \( n \)), using 400 syllables. Probability density functions of each set of syllables were estimated at two different time points (denoted by \( a \) and \( b \)), as \( Q_a \) and \( Q_b \) for the two time points, and the K–L distance score was then calculated to compare the density functions. If we let \( q_a(m, n) \) and \( q_b(m, n) \) denote the estimated probabilities for bin \( (m = 20, n = 5) \) for days \( a \) and \( b \), respectively, then the K–L distance between \( Q_a \) and \( Q_b \) is defined as follows:

\[
D_{KL}(Q_a || Q_b) = \sum_{m=a}^{M} \sum_{n=a}^{N} q_a(m,n) \log_2 \frac{q_a(m,n)}{q_b(m,n)}
\]

A larger value of the K–L distance corresponds to a lower similarity between the distributions of two sets of syllable populations at different time points. Thus, a K–L distance of 0 indicates a perfect match between two sets of syllable populations. Three types of K–L distance were analyzed; (i) K–L distance in a singing cluster, (ii) K–L distance in a resting interval between neighboring singing clusters, and (iii) total K–L distance in a day. (i) The K–L distance in a singing cluster was calculated to detect song changes during
singing, by comparison of scatter density plots of 400 syllables from the beginning and end of a focused singing cluster. (ii) The K–L distance in a resting interval between neighboring singing clusters was calculated to detect song changes during resting. This was achieved by comparing the scatter density plots 400 syllables from the end of a focused singing cluster and the beginning of the following singing cluster. We focused on early, middle, and last singing clusters in diurnal singing to assess the dynamics of song development in a day. The middle singing cluster is located near the middle of a day (6 h after lights were turned on). The earliest (or latest) K–L value in a day was calculated as the distance of syllable populations between the earliest (or latest) 400 syllables of the day and the succeeding (or penultimate) 400 syllables. (iii) Total K–L distance in a day was calculated by the comparison of syllables from the earliest and latest songs in a day as an index of total song change in a day. The change rate in total K–L distance was calculated as

\[
\text{The change rate of a specific singing cluster} = \frac{K-L \text{ distance in a singing cluster}}{\text{Total K–L distance in a day}}
\]

\[
\text{The change rate of a specific resting interval} = \frac{K-L \text{ distance in a resting interval}}{\text{Total K–L distance in a day}}
\]

Manipulation of singing density at juvenile stage

All the birds were raised with their parents and siblings until 40 dph and were then isolated in a sound-attenuating box for song recording. The juvenile birds first were allowed to sing freely and whether the singing density in a day reached a normal high-density score (>5.0 bouts per min) was determined. We then forced the birds (n = 4, 60–67 dph) to sing at a low-singing density (one to five bouts per min) in three singing clusters through a day by occasionally interrupting their singing. The real-time singing behaviors were monitored with Sound Analysis Pro and interrupted by knocking on or opening the sound-attenuating box. On the following day, the juvenile birds were allowed to sing freely at a normal high-singing density, but allowed, by real-time observation, numbers of song bouts and singing clusters (three clusters) similar to those on the singing-interrupted day. The manual song prevention did not lead to abnormal behavioral activities or weight loss in the manipulated birds.
Deafening

The birds were deafened by cochlear extirpation before fledging between 20 and 22 dph. The birds were anesthetized with pentobarbital (6.48 mg/ml; 60 μl/10 g of body weight, Sankyou-Kagaku, Hiratsuka, Japan) by intraperitoneal injection. After the head was fixed in a custom-made stereotaxic apparatus with ear bars, a small window was made through the neck muscle and the skull near the end of the elastic extension of the hyoid bone. A small hole was then made in the cochlear dome. The cochlea was pulled out with a fine hooked wire. The removed cochleae were confirmed by visual inspection under a dissecting microscope. After bilateral cochlear removal, the birds were put back in their nests and kept with their parents and siblings. Examples of song development of the deafened birds were provided in a separate study (Mori and Wada, 2015).
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Author contributions

E.O. and K.W. designed the research. E.O. and C.M. performed the experiments. E.O. and K.W. performed the analysis. C.M. and E.O. developed new analytical methods and tools. E.O. and K.W. wrote the paper.
Figures

Figure 1. Diurnal distribution and frequency of singing in a zebra finch at juvenile and adult stages

(A) Timeline of song learning in the zebra finch.

(B) Number of song bouts per 5 min in a day in the juvenile (54–55 dph, top panel) and adult (101–102 dph, bottom panel) stages. Total number of song bouts each day is shown above. Heat maps under the histograms correspond to singing frequency (bout numbers per 5-min bin).
Figure 2. Developmental change in the diurnal distribution of singing, singing clusters, and singing density

(A) Developmental change of song during the critical period.
(B) Heat maps of singing frequency every 3 days through song development.
(C) Total number of song bouts in a day during the critical period.
(D) Total number of singing clusters in a day. Singing cluster is defined as continuous singing followed by a period of at least 5-min silence (See Materials and methods).
(E) Singing density in all singing clusters in a day (orange dots: singing density in individual singing clusters, red diamonds: median value of singing densities each day).
(F–I) Developmental changes of singing cluster (F), singing bout number per singing cluster (G), singing cluster duration (H), singing density (I) at three stages [early plastic song phase 43–57 dph (red), late plastic song phase 74–87 dph (orange), and crystallized song phase 100–110 dph (yellow)]. $n = 4$ birds, error bars are mean ± SEM. Fisher’s PLSD test, **$P < 0.01$, ***$P < 0.001$. 

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Figure 3. Diurnal oscillation of song development associated with clustered singing in the juvenile stage

(A) Spectrograms of earliest songs (early) and latest songs (late) from a bird at 65 dph. Syllables
with colors are examples used for the calculation of entropy variance (EV, in B).

(B) (Top) 5-min binned histograms of numbers of song bouts from the same bird above at 65 dph. Heat map under the histograms indicates singing frequency as shown in Figure 2B. (Middle) Transition of EVs of individual syllable types. One syllable type (blue line) increases in the value of EV in 1h from initial singing, whereas the other three syllables (green, purple, and light blue lines) maintained similar values of EV throughout the day. Error bars are mean ± SD. (Bottom) Singing-driven dynamics of syllable population. Scatter distribution plots of syllable acoustic features in diurnal songs (syllable duration vs mean frequency modulation (FM) for 400 syllables from each time points). Momentary dynamics of K–L distance from early songs (orange line) and from latest songs (red line). A smaller K–L distance value denotes a distribution similar to that of compared syllable populations.

(C) Diurnal changing rate in total K–L distances of syllable populations in each singing cluster and resting time point in the plastic song phase (45–66 dph). Dots connected with colored lines represent individual birds (n = 9 birds, one-factor ANOVA). Red small horizontal bars represent the average at each time point.
Figure 4. Singing frequency affects oscillation rate of song development in the juvenile stage

(A) Two examples of manipulation of singing frequency. (Upper) Low-frequency singing was enforced in the first experimental day and then followed by normal high-frequency singing on the second experimental day. (Lower) Comparison between the first and latest syllable populations in the day. (Red dots: the earliest 400 syllables in the day; blue dots: the latest 400 song syllables in the day)

(B) Change rate in total K–L distances during singing clusters and resting times under low and high singing frequencies (n = 4 birds, repeated-measure ANOVA, P < 0.001 between low- and high- singing frequency days). Red small horizontal bars represent the average at each time point.
Figure 5. Singing cluster and density and diurnal oscillation of song development in socially isolated and early-deafened birds

(A) Developmental changes of singing cluster and density in socially isolated and early-deafened
birds (juvenile: 39–66 dph, adult: 100–127 dph) (n = 4 birds each, paired t-test, *P < 0.05, **P < 0.01, ***P < 0.001). Red small horizontal bars represent the average of each developmental group.

(B) Change rate in total K–L distances during singing clusters and resting times in socially isolated (upper panel) and early-deafened (lower panel) juveniles (40–64 dph, n = 4 birds each, one-factor ANOVA). Red small horizontal bars represent the average at each time point.

(C) K–L distance between the earliest and latest song populations in control intact, socially isolated and early-deafened juveniles (n = 9, 4, and 4 birds, respectively, Scheffé's F test, **P < 0.01).
References


