© 2015. Published by The Company of Biologists Ltd | The Journal of Experimental Biology (2015) 218, 2260-2268 doi:10.1242/jeb.115105

RESEARCH ARTICLE

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

Eri Ohgushi[1], Chihiro Mori[1] and Kazuhiro Wada[1,2,3,*]

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 with low frequency in short clusters. This juvenile-specific clustered singing was characterized by clear separations of daily time for intense 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 the day. When the singing frequency was manipulated to decrease it at the juvenile stage, the oscillation rate of song development was dramatically reduced. Although the juvenile-specific clustered singing occurred in non-tutored socially isolated birds or those with auditory deprivation, the diurnal oscillation of vocal development was only observed in non-tutored 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.

KEY WORDS: Learned vocalization, Sensorimotor learning, Zebra finch, Self-motivated behavior

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 in terms of trial number, 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 mature 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 (Derégnaucourt 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 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 the stability of the syllable sequence (Johnson et al., 2002). This correlation suggests the importance of an appropriate amount of singing 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 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 with by disturbance of dense singing or by a 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 number of song

[1]Graduate School of Life Science, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan. [2]Department of Biological Sciences, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan. [3]Faculty of Science, Hokkaido University, Sapporo, Hokkaido 060-0810, Japan.

*Author for correspondence (wada@sci.hokudai.ac.jp)

Received 6 October 2014; Accepted 12 May 2015

The Journal of Experimental Biology

The Journal of Experimental Biology (2015) 218, 2260-2268 doi:10.1242/jeb.115105

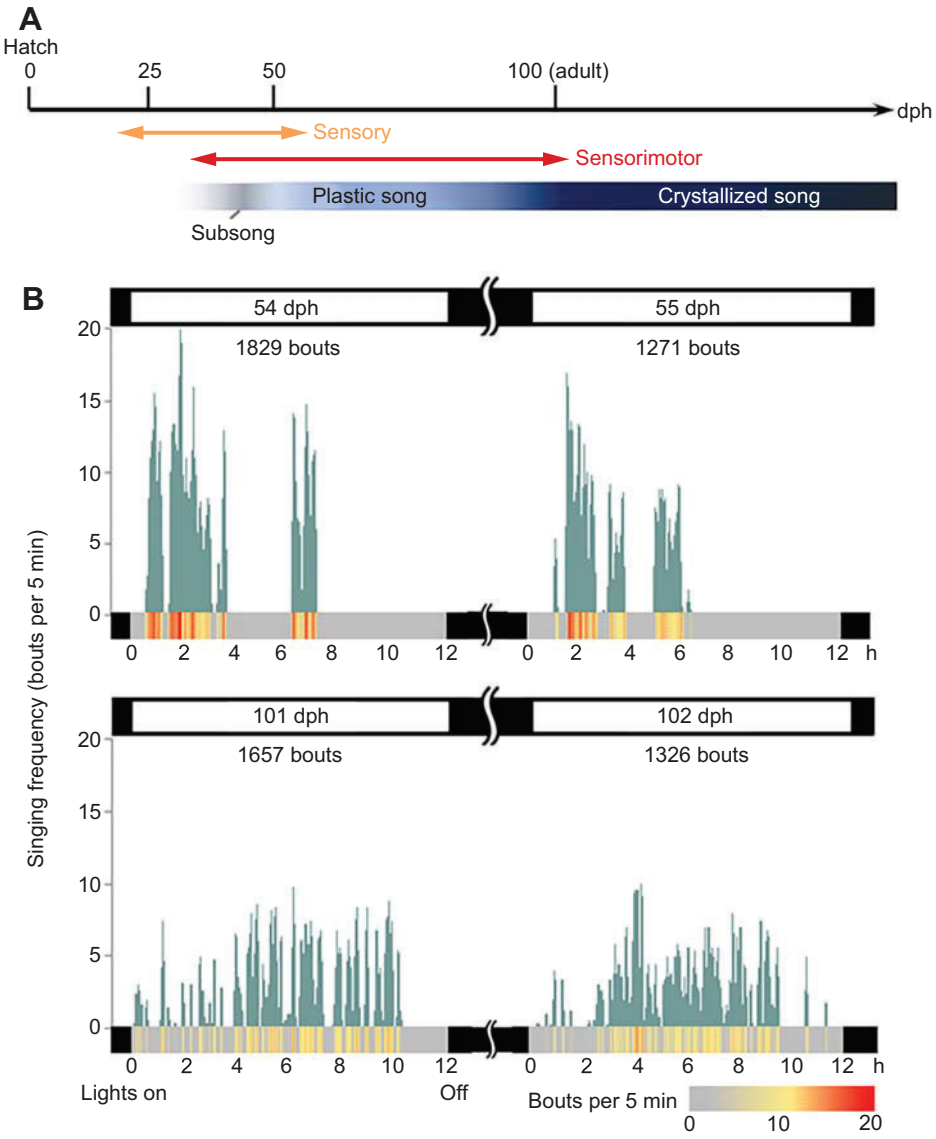

Fig. 1. Diurnal distribution and frequency of singing in a zebra finch at juvenile and adult stages. (A) Timeline of song learning in the zebra finch. (B) Number of song bouts per 5 min in a day in the juvenile (54–55 dph, top panel) and adult (101–102 dph, bottom panel) stages. The total number of song bouts each day is shown at the top of each panel. Heat maps under the histograms correspond to singing frequency (number of bouts per 5 min bin). dph, days post-hatch.

bouts per 5 min bin over two consecutive days was calculated for both stages and is represented as a singing histogram (Fig. 1B, blue) and heat map (Fig. 1B, gray to red). The total number of diurnal singing bouts in the juvenile stage was almost the same as 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 production 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 of our zebra finches (N=4) from 28 to 40 dph, when juveniles began to sing until their songs were crystallized at 105–110 dph. We then analyzed the distribution of song production, bout number and daily frequency of singing per 5 min bin throughout the song developmental period. Sonograms and heat maps of singing frequency depicted the developmental changes in diurnal song production through the critical period (e.g. Fig. 2A,B). High-frequency singing (Fig. 2B, red) was observed at approximately 50–75 dph, when the birds 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 number 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 singing cluster as a continuous session of singing that was preceded by more than 5 min of silence (no singing), and singing density as the frequency of singing in a singing cluster (calculated as the number of singing bouts/singing cluster duration). The number of singing clusters gradually increased from 70 dph to the adult stage (Fig. 2D,F). In contrast, the diurnal singing density showed an inverse distribution relative to the diurnal distribution of singing clusters through development (Fig. 2E,G). The singing density showed a large variation among singing clusters in a day, although the median 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,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

The Journal of Experimental Biology (2015) 218, 2260-2268 doi:10.1242/jeb.115105

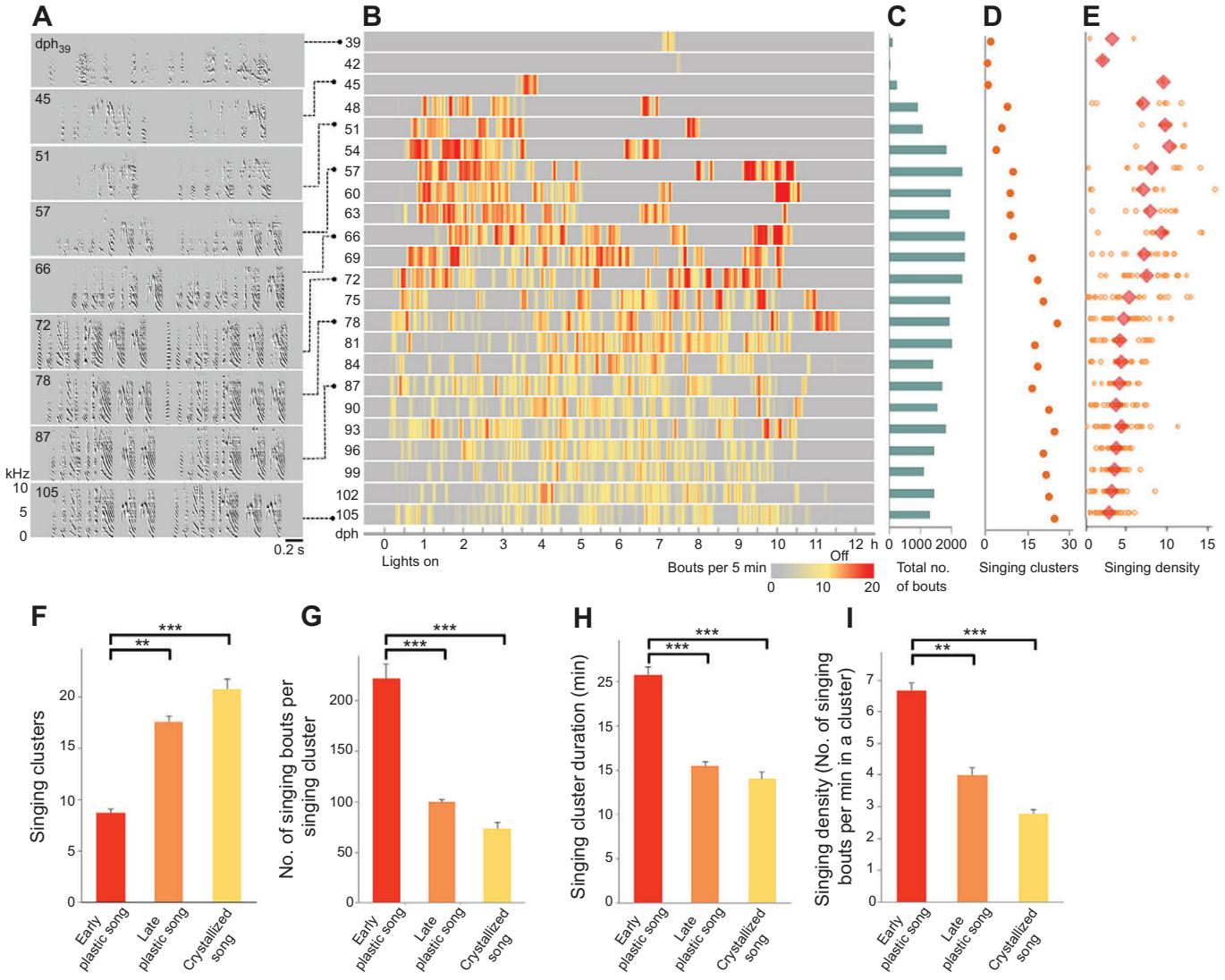

Fig. 2. Developmental change in the diurnal distribution of singing, singing clusters and singing density. (A) Developmental change of song during the critical period. (B) Heat maps of singing frequency every 3 days through song development. (C) Total number of song bouts in a day during the critical 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 density each day). (F–I) Developmental changes of singing cluster (F), number of singing bouts per singing cluster (G), singing cluster duration (H) and singing density (I) at three stages (early plastic song phase, 43–57 dph; late plastic song phase, 74–87 dph; and crystallized song phase, 100–110 dph). *N*=4 birds, means+s.e.m. Fisher's PLSD test, $^{**}P<0.01$, $^{***}P<0.001$.

late plastic song phase (74–87 dph) and the crystallized song phase (100–110 dph). Four singing phenotypes were analyzed here: singing cluster, number of singing bouts per singing cluster, singing cluster duration 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, $P<0.01$ to 0.001). This indicated that zebra finches in the early plastic song phase produced high singing density in sporadic but long singing clusters, generating spaced practice time and rest within 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 (Derégnaucourt et al., 2005; Deshpande et al., 2014). Accordingly, we first calculated the diurnal transitions in the EV of syllables at the beginning and end of each singing cluster and a time point 3–4 h after lights were turned on. As previously reported (Derégnaucourt et al., 2005), the EV of some but not all syllables dramatically changed within only a few hours of the onset of diurnal singing (Fig. 3A,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

The Journal of Experimental Biology (2015) 218, 2260-2268 doi:10.1242/jeb.115105

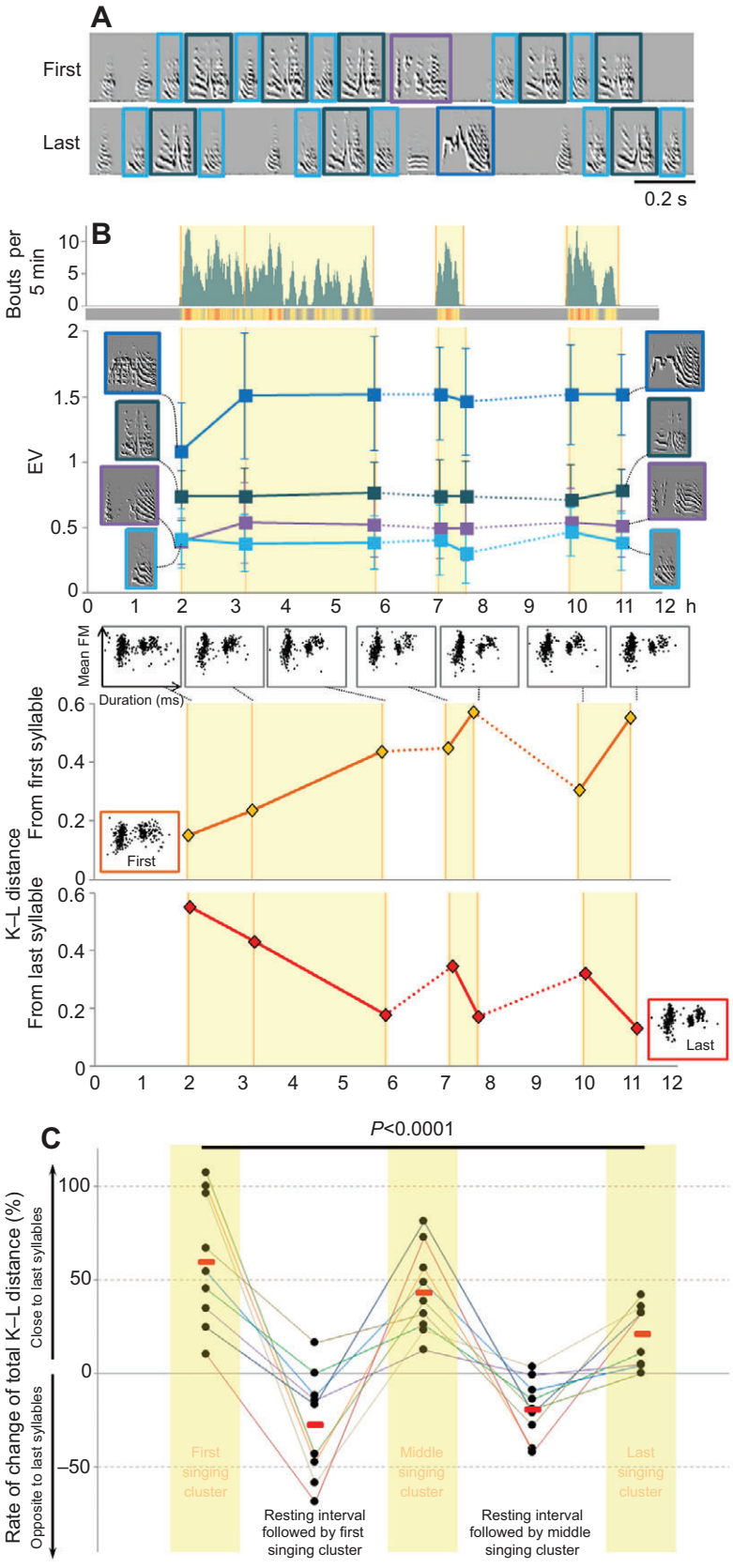

Fig. 3. Diurnal oscillation of song development associated with clustered singing in the juvenile stage.
(A) Spectrograms of first and last songs from a bird at 65 dph. The boxed syllables are examples used for the calculation of entropy variance (EV, in B). (B) The top panel shows the 5 min binned histograms of the number of song bouts from the same bird above at 65 dph. The heat map under the histograms indicates singing frequency as shown in Fig. 2B. The middle panel shows the transition of EVs of individual syllable types. One syllable type (blue line) increases in EV value 1 h from initial singing, whereas the other three syllables (green, purple and light blue lines) maintained similar values of EV throughout the day. Means±s.d. The bottom panel shows singing-driven dynamics of the syllable population. Scatter distribution plots of syllable acoustic features in diurnal songs [syllable duration versus mean frequency modulation (FM) for 400 syllables from each time point] are given at the top. Momentary dynamics of the Kullback–Leibler (K–L) distance from the first songs (orange line) and from the last songs (red line) are shown below. A smaller K–L distance value denotes a distribution similar to that of compared syllable populations. (C) Diurnal rate of change in total K–L distance 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). Small red horizontal bars represent the average at each time point.

development. We used information from two acoustic parameters and the population rate of produced syllables, given that the juveniles must change multiple parameters of song features, not just 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

The Journal of Experimental Biology (2015) 218, 2260-2268 doi:10.1242/jeb.115105

apparent differences between distributions of the first and last 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 from that 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 calculating 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 first syllable population (Fig. 3B, orange) or the last syllable population in a day (Fig. 3B, red). In comparison with the first syllable population, a divergence of syllable density population was induced during singing clusters and a regression of transition toward the first syllable population occurred during resting times between singing clusters (Fig. 3B, orange). Symmetrical diurnal dynamics of the syllable population was observed in comparison with the last syllable population of the day (Fig. 3B, red). 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 last 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 last 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 were regulated with an inverse relationship during development. Thus, juveniles generate songs with a high frequency in sporadic singing clusters at the early plastic song phase (Fig. 2F,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 shows two examples). As a result, birds produced lower singing frequency in any cluster in the experimental day compared with a subsequent high (normal) singing frequency day (average singing density in the day, 2±0.3 and 7±0.9, respectively, means±s.e.m.). Diurnal change in the syllable populations was then assessed by comparing the first and last syllable populations in the two successive days. The oscillation of the 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 compared with 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 contribute to the regulation of clustered singing and diurnal oscillation of song development. For this purpose, juvenile birds were placed under two conditions, non-tutored social isolation or auditory deprivation. We compared their diurnal song production at two developmental stages, juvenile (39–66 dph) and adult (100–127 dph). The two experimental procedures started before the sensory learning period for vocal learning (see Materials and methods). The non-tutored birds in solitary isolation exhibited a tendency for generating 'parts' of a motif structure with prolonged and variable syllables. However, the time point of appearance of stabilized acoustic features for the majority of the syllables was similar to that in the normal birds. In contrast, the birds with auditory deprivation 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 stable temporal sequences of distinguishable syllables by approximately 300 dph (Mori and Wada, 2015). Under both rearing 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 to 0.001). In terms of the number of singing clusters, both socially isolated birds and those with auditory deprivation showed an increasing trend through development as observed in live-tutored birds (Fig. 2F). Conversely, singing density decreased through development, as similarly represented in live-tutored birds (Fig. 2I). Furthermore, the socially isolated birds and those with auditory deprivation showed a similar number of singing clusters and a similar singing density to 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 during 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 non-tutoring social isolation and auditory deprivation conditions, the diurnal oscillation of vocal development was only observed in socially isolated juveniles (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 first and last songs in a single day, we found a significant difference in diurnal song development rate between normal intact and auditory-deprived 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

The Journal of Experimental Biology (2015) 218, 2260-2268 doi:10.1242/jeb.115105

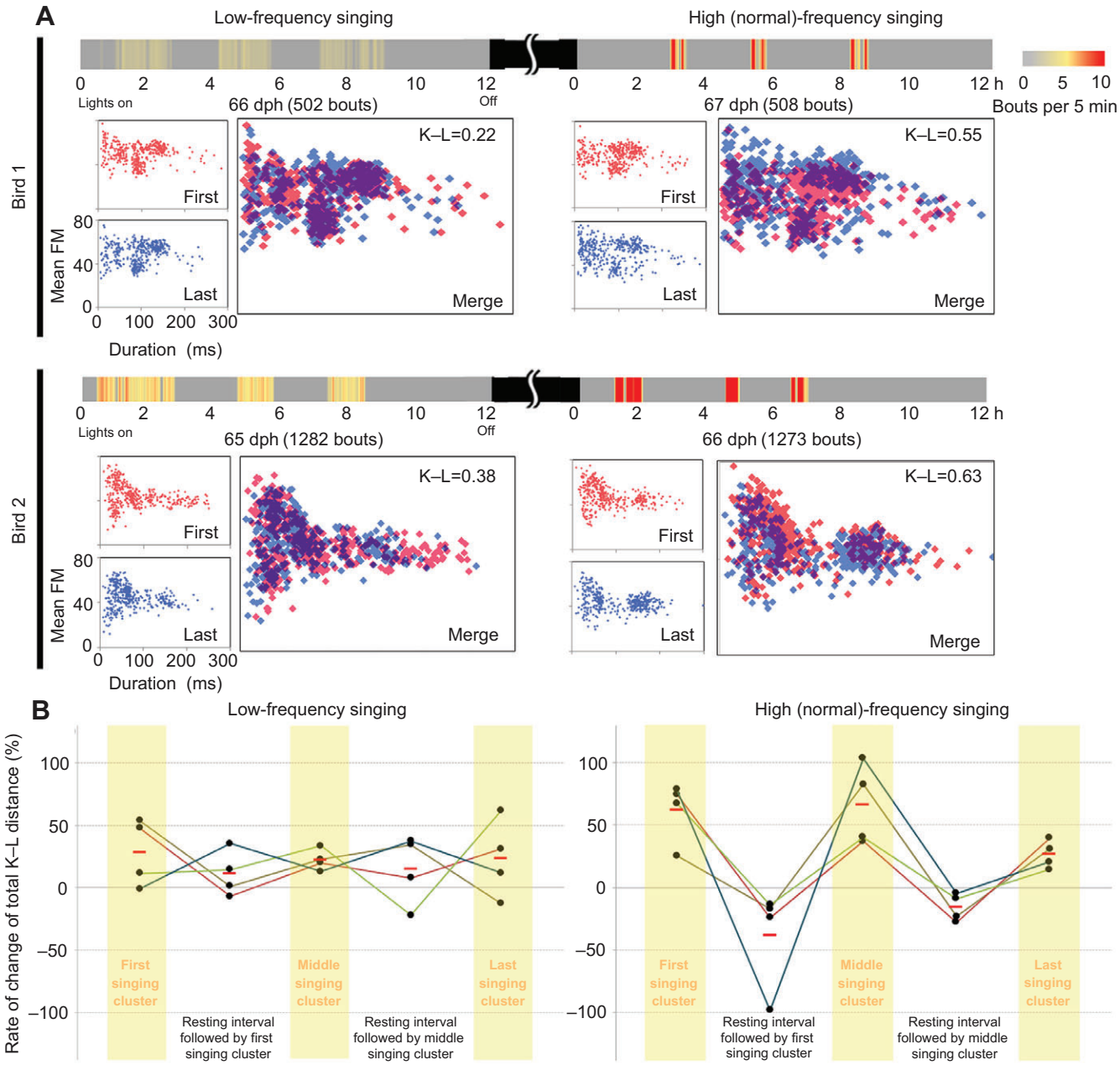

Fig. 4. Singing frequency affects oscillation rate of song development in the juvenile stage. (A) Two examples of manipulation of singing frequency. Top: low-frequency singing was enforced in the first experimental day (left) and then followed by normal high-frequency singing on the second experimental day (right). Bottom: comparison between the first and last syllable populations in the day. Red dots indicate the first 400 syllables in the day; blue dots, the last 400 song syllables in the day. (B) Rate of change of total K–L distance during singing clusters and resting times with low and high (normal) singing frequencies [N=4 birds, repeated-measures ANOVA, P<0.001 between low and high (normal) singing frequency days]. Small red horizontal bars represent the average at each time point.

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 decreased by experimental manipulation, without affecting rendition clusters and the total amount of singing, 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 (Derégnaucourt et al., 2005). Although we speculated that the observed diurnal oscillation of syllable acoustics may have been affected by daytime naps during the singing rests, the juveniles in this study actually 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 (Derégnaucourt et al., 2005). 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 a similarity score to

The Journal of Experimental Biology (2015) 218, 2260-2268 doi:10.1242/jeb.115105

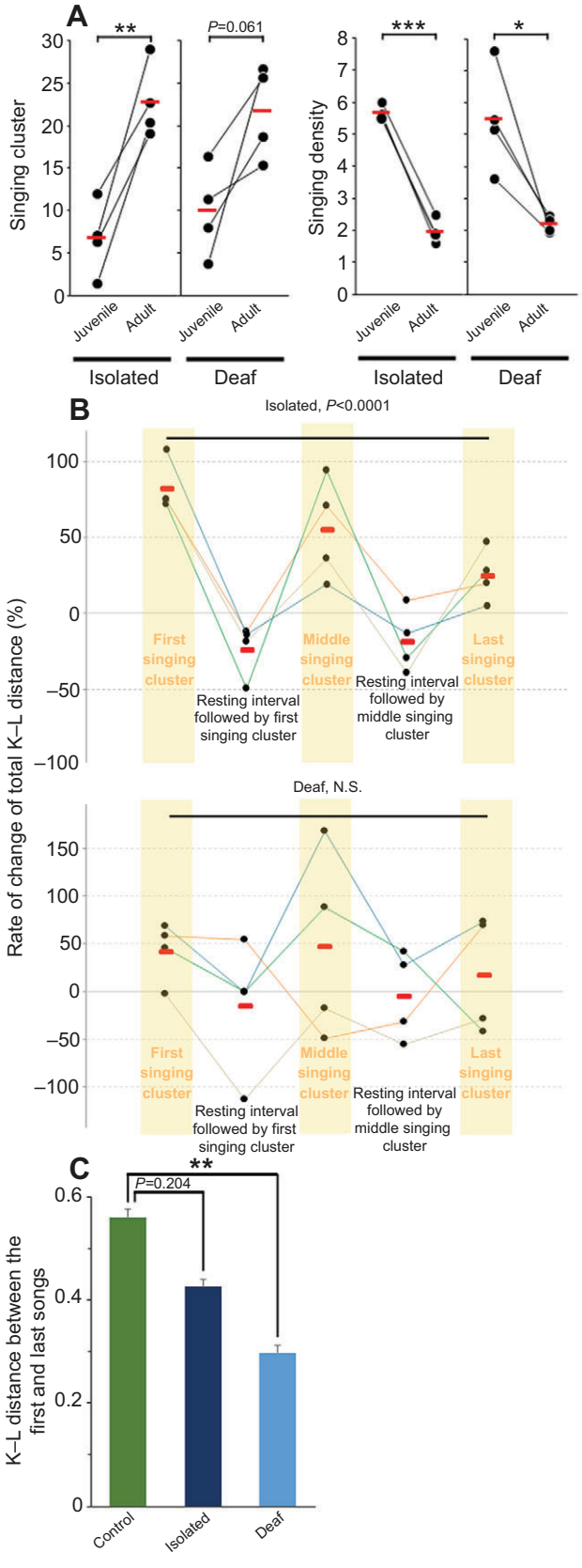

Fig. 5. Singing cluster and density and diurnal oscillation of song development in socially isolated birds and those with auditory deprivation. (A) Developmental changes of singing cluster and density in socially isolated and deaf birds (juvenile, 39–66 dph; adult, 100–127 dph) (N=4 birds each, paired t-test, *P<0.05, **P<0.01, ***P<0.001). Small red horizontal bars represent the average of each developmental group. (B) Rate of change of total K–L distance during singing clusters and resting times in socially isolated (upper panel) and deaf (lower panel) juveniles (40–64 dph, N=4 birds each, one-factor ANOVA). Small red horizontal bars represent the average at each time point. (C) K–L distance between the first and last song populations in control intact, socially isolated and deaf juveniles (N=9, 4 and 4 birds, respectively, Scheffé's F-test, **P<0.01).

the song model in the previous study (Derégnaucourt et al., 2005) 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 of the onset of diurnal singing (Fig. 3B), it would be highly conceivable that a different acoustics parameter changes with a different time course for 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 combined results suggest the existence of an hourly and daily 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 non-verbal 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 previous studies were based on the observation of well-controlled enforced tasks for which trial number and duration 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 impact of spaced training that is voluntarily generated during developmental learning. In this study, we found that manipulation of singing inhibition, social isolation or auditory deprivation does not affect the development of clustered singing, indicating an intrinsic regulation of singing frequency and timing throughout the critical period of vocal development. However, singing-inhibited or deaf juveniles did not show distinct diurnal oscillation of song development. In contrast, socially isolated (non-tutored) 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 the 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 is 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, Fig. 2C). In contrast, the diurnal frequency and timing of singing were differentially regulated during development in an intrinsic manner (as observed for socially isolated and deaf birds in a sound attenuation box), suggesting that neural substrates innately regulate the developmental change of singing frequency and timing in a day.

The Journal of Experimental Biology (2015) 218, 2260-2268 doi:10.1242/jeb.115105

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 (K.W., unpublished data). 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 is 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; Riters 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 phenotype 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 a similar total singing duration (Jin and Clayton, 1997; Wada et al., 2006; Whitney et al., 2000). However, the factors directly regulating distinct expression 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 AND 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 frequency of singing in a singing cluster [number of song bouts/singing cluster duation (min)].

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 h:11 h light:dark cycle. During song recording sessions, each bird was individually housed in a sound-attenuating box. For the analysis of singing frequency during 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. Birds in solitary isolation were transferred with their mother into a sound-attenuating box before 5 dph and kept away 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 isolated 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, IL, USA) connected to a computer with 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 EV 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 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 (in ms; denoted by m) and mean FM (denoted by 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 the bin (m=20, n=5) for days a and b, respectively, then the K–L distance (D_{K-L}) between Q_a and Q_b is defined as follows:

$$D_{K-L}(Q_a||Q_b) = \sum_{m=a}^{M}\sum_{n=a}^{N} q_a(m,n)\log_2\frac{q_a(m,n)}{q_b(m,n)}. \quad (1)$$

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 analysed: (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 the first, 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 first (or last) 400 syllables of the day and the succeeding (or penultimate) 400 syllables. (iii) Total K–L distance in a day was calculated by comparison of syllables from the first and last songs in a day as an index of total song change in a day. The rate of change in total K–L distance was calculated as:

$$\text{Rate of change of a specific singing cluster} = \frac{\text{(i) K}-\text{L distance in a singing cluster}}{\text{(iii) Total K}-\text{L distance in a day}}, \quad (2)$$

$$\text{Rate of change of a specific resting interval} = \frac{\text{(i) K}-\text{L distance in a resting interval}}{\text{(iii) Total K}-\text{L distance in a day}}. \quad (3)$$

Manipulation of singing density at the 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 were first allowed to sing freely to determine whether the singing density in a day reached a normal high-density score (>5.0 bouts min^{-1}). We then forced the birds (N=4, 60–67 dph) to sing at a low singing density (1–5 bouts min^{-1}) in three singing clusters over 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 could sing freely at a normal high singing density but were allowed, by real-time observation, a similar number of song bouts and singing clusters (three clusters) to those on the previous, singing-interrupted day. This manual prevention of singing did not lead to abnormal behavioral activities or weight loss in the manipulated birds.

Auditory deprivation

The birds in this group underwent cochlear extirpation before fledging between 20 and 22 dph. The birds were anesthetized with pentobarbital (6.48 mg ml^{-1}; 60 µl/10 g of body mass; Sankyou-Kagaku, Hiratsuka, Japan) by intraperitoneal injection. The head was fixed in a custom-made stereotaxic apparatus with ear bars, and 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 returned to their nests and kept with their parents and siblings. Examples of song development of the deaf birds are provided in a separate study (Mori and Wada, 2015).

Acknowledgements

We would like to thank R. Imai and S. Hayase and Dr H. Horita for their critical comments. We thank Dr M. Soma for help with statistical analysis.

Competing interests

The authors declare no competing or financial interests.

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.

Funding

This work was supported by a Grant-in-Aid for the Japan Society for the Promotion of Science (JSPS) Fellows 11J07071 (to C.M.) and from the Uehara Memorial Foundation, The Asahi Glass Foundation, The Sumitomo Foundation, and JSPS KAKENHI grant number 25290063 and 25640097 (to K.W.).

References

Bramham, C. R. (2008). Local protein synthesis, actin dynamics, and LTP consolidation. *Curr. Opin. Neurobiol.* **18**, 524-531.

Bramham, C. R., Worley, P. F., Moore, M. J. and Guzowski, J. F. (2008). The immediate early gene arc/arg3.1: regulation, mechanisms, and function. *J. Neurosci.* **28**, 11760-11767.

Carulli, D., Foscarin, S. and Rossi, F. (2011). Activity-dependent plasticity and gene expression modifications in the adult CNS. *Front. Mol. Neurosci.* **4**, 50.

Clayton, D. F. (2000). The genomic action potential. *Neurobiol. Learn. Mem.* **74**, 185-216.

Derégnaucourt, S., Mitra, P. P., Fehér, O., Pytte, C. and Tchernichovski, O. (2005). How sleep affects the developmental learning of bird song. *Nature* **433**, 710-716.

Deshpande, M., Pirlepesov, F. and Lints, T. (2014). Rapid encoding of an internal model for imitative learning. *Proc. R. Soc. B Biol. Sci.* **281**, 20132630.

Dudai, Y. and Eisenberg, M. (2004). Rites of passage of the engram: reconsolidation and the lingering consolidation hypothesis. *Neuron* **44**, 93-100.

Ebbinghaus, H. (1885). *Über das Gedächtnis. Untersuchungen zur experimentellen Psychologie [Memory: A Contribution to Experimental Psychology]*. Leipzig, Germany: Duncker & Humblot.

Immelmann, K. (1969). Song development in the zebra finch and other estrildid finches. In *Bird Vocalizations* (ed. R. Hinde), pp. 61-74. Cambridge: Cambridge University Press.

Jin, H. and Clayton, D. F. (1997). Localized changes in immediate-early gene regulation during sensory and motor learning in zebra finches. *Neuron* **19**, 1049-1059.

Johnson, F., Soderstrom, K. and Whitney, O. (2002). Quantifying song bout production during zebra finch sensory-motor learning suggests a sensitive period for vocal practice. *Behav. Brain Res.* **131**, 57-65.

Khurshid, N., Jayaprakash, N., Hameed, L. S., Mohanasundaram, S. and Iyengar, S. (2010). Opioid modulation of song in male zebra finches (*Taenopygia guttata*). *Behav. Brain Res.* **208**, 359-370.

Menzel, R., Manz, G., Menzel, R. and Greggers, U. (2001). Massed and spaced learning in honeybees: the role of CS, US, the intertrial interval, and the test interval. *Learn Mem.* **8**, 198-208.

Mori, C. and Wada, K. (2015). Audition-independent vocal crystallization associated with intrinsic developmental gene expression dynamics. *J. Neurosci.* **35**, 878-889.

Naqib, F., Sossin, W. S. and Farah, C. A. (2012). Molecular determinants of the spacing effect. *Neural Plast.* **2012**, 581291.

Okamoto, T., Endo, S., Shirao, T. and Nagao, S. (2011). Role of cerebellar cortical protein synthesis in transfer of memory trace of cerebellum-dependent motor learning. *J. Neurosci.* **31**, 8958-8966.

Pagani, M. R., Oishi, K., Gelb, B. D. and Zhong, Y. (2009). The phosphatase SHP2 regulates the spacing effect for long-term memory induction. *Cell* **139**, 186-198.

Riters, L. V., Schroeder, M. B., Auger, C. J., Eens, M., Pinxten, R. and Ball, G. F. (2005). Evidence for opioid involvement in the regulation of song production in male European starlings (*Sturnus vulgaris*). *Behav. Neurosci.* **119**, 245-255.

Rudenko, A., Dawlaty, M. M., Seo, J., Cheng, A. W., Meng, J., Le, T., Faull, K. F., Jaenisch, R. and Tsai, L.-H. (2013). Tet1 is critical for neuronal activity-regulated gene expression and memory extinction. *Neuron* **79**, 1109-1122.

Tang, S. J., Reis, G., Kang, H., Gingras, A.-C., Sonenberg, N. and Schuman, E. M. (2002). A rapamycin-sensitive signaling pathway contributes to long-term synaptic plasticity in the hippocampus. *Proc. Natl. Acad. Sci. USA* **99**, 467-472.

Tchernichovski, O., Nottebohm, F., Ho, C. E., Pesaran, B. and Mitra, P. P. (2000). A procedure for an automated measurement of song similarity. *An. Behav.* **59**, 1167-1176.

Tchernichovski, O., Mitra, P. P., Lints, T. and Nottebohm, F. (2001). Dynamics of the vocal imitation process: how a zebra finch learns its song. *Science* **291**, 2564-2569.

Tully, T., Preat, T., Boynton, S. C. and Del Vecchio, M. (1994). Genetic dissection of consolidated memory in *Drosophila*. *Cell* **79**, 35-47.

Vlach, H. A., Sandhofer, C. M. and Kornell, N. (2008). The spacing effect in children's memory and category induction. *Cognition* **109**, 163-167.

Wada, K., Howard, J. T., McConnell, P., Whitney, O., Lints, T., Rivas, M. V., Horita, H., Patterson, M. A., White, S. A., Scharff, C. et al. (2006). A molecular neuroethological approach for identifying and characterizing a cascade of behaviorally regulated genes. *Proc. Natl. Acad. Sci. USA* **103**, 15212-15217.

Wahlheim, C. N., Dunlosky, J. and Jacoby, L. L. (2011). Spacing enhances the learning of natural concepts: an investigation of mechanisms, metacognition, and aging. *Mem. Cognit.* **39**, 750-763.

West, A. E. and Greenberg, M. E. (2011). Neuronal activity–regulated gene transcription in synapse development and cognitive function. *Cold Spring Harb. Perspect. Biol.* **3**, a00574.

Whitney, O., Soderstrom, K. and Johnson, F. (2000). Post-transcriptional regulation of zenk expression associated with zebra finch vocal development. *Mol. Brain Res.* **80**, 279-290.

Wu, W., Thompson, J. A., Bertram, R. and Johnson, F. (2008). A statistical method for quantifying songbird phonology and syntax. *J. Neurosci. Methods* **174**, 147-154.

Zann, R. (1996). *The Zebra Finch: A Synthesis of Field and Laboratory Studies*. Oxford: Oxford University Press.