Silent songs: What birds rehearse during sleep

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Brain activity during sleep plays an important role in memory consolidation, including motor memory. One suggested mechanism of how neural activity effects these benefits is through reactivation of neurons in patterns resembling those of the preceding experience. It is largely unknown for any system, which specific patterns of motor activation are replayed during sleep. Brain areas devoted to song production in the songbird brain exhibit spontaneous song-like activity during sleep, but single cell neural recordings did not permit detection of the specific song patterns. We have now discovered that this sleep activation can be detected in the muscles of the vocal organ, thus providing a unique window into song-related brain activity at night. We show that male zebra finches frequently exhibit spontaneous song-like activity during the night, but that the fictive song patterns are highly variable and uncoordinated compared to the highly stereotyped day-time song production. This substantial variability is not consistent with the idea that night-time activity replays day-time experiences for consolidation, but may instead serve to generate internal error signals that help maintain the high stereotypy of day-time song. This alternative function of sleep motor activity may apply more generally to other behaviors, including human dream-related motor activity. rV

1 **Title:**   **Silent songs: What birds rehearse during sleep**

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15 **Key words:** motor replay, sleep, song, motor program, consolidation

17   **Abstract:** Brain activity during sleep plays an important role in memory consolidation, including

18   motor memory. One suggested mechanism of how neural activity effects these benefits is

19   through reactivation of neurons in patterns resembling those of the preceding experience. It is

20   largely unknown for any system, which specific patterns of motor activation are replayed during

21   sleep. Brain areas devoted to song production in the songbird brain exhibit spontaneous song-like

22   activity during sleep, but single cell neural recordings did not permit detection of the specific

23   song patterns. We have now discovered that this sleep activation can be detected in the muscles

24   of the vocal organ, thus providing a unique window into song-related brain activity at night. We

25   show that male zebra finches frequently exhibit spontaneous song-like activity during the night,

26   but that the fictive song patterns are highly variable and uncoordinated compared to the highly

27   stereotyped day-time song production. This substantial variability is not consistent with the idea

28   that night-time activity replays day-time experiences for consolidation, but may instead serve to

29   generate internal error signals that help maintain the high stereotypy of day-time song. This

30   alternative function of sleep motor activity may apply more generally to other behaviors,

31   including human dream-related motor activity.

33 **Introduction**

34 Brain activity during sleep is thought to play an important role in consolidation of declarative

35 and procedural memory (e.g., Maquet, 2001; Stickgold, 2005; Diekelmann & Born, 2010; Yang

36 et al., 2014; Tononi & Cirelli, 2014), and one suggested mechanism of how neural activity

37 effects these benefits is through reactivation of neurons in patterns that resemble those of the

38 preceding experience during the active period (Wilson & McNaughton, 1994; Dave &

39 Margoliash, 2000; Hahnloser, Kozhevnikov & Fee, 2002; 2006; Euston, Tasuno & McNaughton,

40 2007; Peyrache et al., 2009; Shank & Margoliash, 2009). Specifically, motor replay during sleep

41 can be induced by sensory cues and is thought to consolidate motor memory through

42 sensorimotor re-processing in the relevant brain areas (Derégnaucourt et al., 2005; Margoliash,

43 2005; Orban et al., 2006; Hupbach et al., 2009). However, the role of sleep on memory formation

44 for a specific newly acquired motor skill is debated (e.g., Korman et al., 2003; Rickard et al.,

45 2008; Brawn, Nusbaum & Margoliash, 2010). Furthermore, alternative patterns of sleep

46 activation have been reported and led to different, debated hypotheses regarding the function of

47 this brain activity (e.g., Dragoi & Tonegawa, 2011; 2013; Brawn & Margoliash, 2015;

48 Eichenbaum, 2015; Silva, Feng & Foster, 2015; Grosmark & Buzsáki, 2016).

49 This possibility of multiple functions of sleep activation patterns is also suggested by the

50 occurrence of replay in well-established and stereotyped motor behaviors (Dave & Margoliash,

51 2000; Margoliash & Schmidt, 2010). Mechanisms for how sleep activation enhances

52 consolidation of recently acquired memories may be different from those involved in stereotyped

53 motor sequences, but this distinction has not been clearly made. In general, our understanding of

54 how night-time motor replay in the brain can benefit motor performance is impeded by the fact

55 that we do not have detailed insight into the nature of the replayed motor patterns.

56   One of the best postulated examples for motor replay is learned vocal behavior (Giret,

57   Edeline & DelNegro, 2017). In birds, activity of neurons in sensorimotor motor (HVC) and

58   motor cortical (the robust arcopallial nucleus, RA) areas strongly suggested that they were

59   activated in song-like manner at night during song playback as well as spontaneously (Dave &

60   Margoliash, 2000; Hahnloser, Kozhevnikov & Fee, 2002; 2006). However, constraints on

61   recording from a large number of neurons simultaneously and on maintaining single unit

62   responses over sufficient time spans did not permit detection of which song sequences are

63   replayed at night. Birdsong is a learned behavior, whose control involves coordination of

64   multiple motor systems to generate a highly stereotyped acoustic sequence (Brainard, 2008;

65   Goller & Cooper, 2008; Suthers & Zollinger, 2008; Méndez et al., 2010; Riede & Goller, 2010;

66   Beckers, 2013). Insight into the specific motor patterns of night-time brain activation would

67   therefore be a major advance in our understanding of how activity during sleep might benefit

68   stereotyped motor control of complex behavior. Here we use song-like night-time activation of

69   the muscles controlling the vocal organ of birds as a sufficiently detailed output to allow

70   identification of which song syllable sequence is replayed in the brain during night-time.

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Material and methods

73   During this study ten adult male zebra finches were used (age >120 days). They were

74   housed individually in a 31.8 cm x 22.9 cm x 27.9 cm wire cage with newspaper lining. They

75   were fed a mixture of red and white millet, canary seed, and water *ad libitum*. This diet was

76   supplemented with peas and corn every other day. Before any surgical procedure was performed,

77   a baseline recording of song was obtained using a directional microphone (Audiotechnica 835B)

78   placed approximately 20 cm away from the bird. The microphone output was amplified using a

79   Brownlee 410 amplifier (200-400x; Brownlee Precision) and connected to an analog to digital

80   converter (National Instruments). Recordings were obtained using Avisoft-Recorder (Avisoft

81   Bioacoustics) at 44.1 kHz sample rate.

82   Zebra finches were accustomed to the procedures three to five days before the surgery by

83   first attaching a leash and backpack to the bird. The backpack is custom-built and consists of a

84   Velcro tab on the back that is secured on the bird with elastic bands around the base of each wing

85   and around the upper part of the thorax. Birds were tethered by a wire, which was fixed on the

86   backpack and connected to a balancing lever arm positioned above the cage to allow the bird to

87   move freely. Once birds resumed singing activity on the tether, surgical procedures were

88   initiated. One hour prior to surgery, birds were deprived of food and water.

89   Surgery was performed under general anesthesia by administering Ketamine/Xylazine.

90   During surgery, a flexible cannula was inserted into a thoracic air sac. The cannula was sutured

91   and adhered (Vetbond; 3M Animal Care Products) to the rib cage and attached to a piezoresistive

92   pressure transducer (Fujikura FPM-02PG) mounted on the backpack. Next, the syrinx was

93   accessed by a skin incision in the furcula area and opening of the interclavicular air sac

94   membrane. The tips of custom-built bipolar wire electrodes (California Fine Wire, SS 304; 25

95   μm) were inserted into the syringeal muscles. Bipolar electrodes were placed in two different

96   muscles during surgery. In each bird, we recorded from either 2 different muscles on the same

97   side, or from left and right muscles (Fig. S1; Table S1). The electrodes were adhered to the

98   surface of the syringeal muscles with tissue adhesive (Vetbond). Electrode wires were routed

99   subcutaneously to the backpack where the ends were attached to a connector from which larger

100   wires made connection to signal conditioning equipment. Following surgery, the bird was placed

101   in the cage, tethered, and allowed to recover overnight.

102   Following surgery, four channels were recorded simultaneously using Avisoft-Recorder,

103   air sac pressure, two EMGs, and audio. EMG channels were band pass filtered (100-3000 Hz)

104   with a gain of 1000x-3000x (Brownlee Precision, 440). Audio recording was done with the same

105   settings that were used to record pre-surgery song. Prior to any nighttime recording, we obtained

106   multiple examples of complete, directed song from the male during the day by placing a female

107   in a cage next to him. The spectrograms from these songs were compared to those recorded pre-

108   surgery to ensure that no damage was done to the syringeal muscles during surgery.

109   During the night, all channels were continuously recorded at a sample rate of 8 kHz.

110   Recordings were started and stopped manually. The photoperiod was set from 6:00 AM to 8:00

111   PM and was controlled by an automatic timer.

112   Comparison of spontaneous EMG activity to EMG activity that occurred during song was

113   done using custom written software implementing the following procedures. The simultaneous

114   measurement of air sac pressure and muscle activity (EMG) for singing birds was used to

115   segment the latter into syllable-related fragments. We computed the envelopes of the EMG

116   signals, and those smoothed time series were cut at specific time points, chosen in the following

117   way. The minima of the pressure patterns with subatmospheric values of air sac pressure were

118   computed, and then, for each minimum, we looked for the immediately following minimum of

119   the simultaneously measured EMG envelope. Those times were used to cut the EMG envelope in

120   N segments, corresponding to the each syllable of the song. These EMG segments corresponding

121   to syllables are hereafter called syllable templates.

122   The envelopes of EMG signals were computed by applying a Hilbert transform (impulse

123   response filter length IRFL=128), followed by a first order integration ($\tau_{\iota\nu\tau}$=0.01), and a Savitsky

124   Golay filter (Nleft=256, Nright=256). We applied this procedure to the syllable templates, as

125 well as to the EMG data recorded during the night (*n(t)*). Then, we computed the correlation

126 between the envelope of the night data *n(t)* and the envelope of each of the syllable templates,

127 shifted at delays τ between 0 and *T-T_{template}*, where *T* is the duration of the night recording and

128 *T_{template}* the duration of the template analyzed. In this way, for each of the templates, we obtained

129 a continuous time series whose largest peaks indicate a high correlation between a fragment of

130 the envelope of the night data, and the syllable template under analysis. In order to identify the

131 largest correlation values, we computed for each bird, all the maxima of the correlation

132 coefficients with all the templates. Then, we fitted a Gaussian G (μ,σ) to the resulting histogram,

133 and defined the threshold value X such that $Z=X-\mu/\sigma=1.64$. With this choice of threshold,

134 every segment of the envelope of the night activity *n(t)*, whose correlation with a template

135 is higher than X, was found to correspond to a segment of the time series in which activity

136 was found (no false positives), and was similar to the template. To test the automated

137 search algorithm, we compared EMG patterns of syllables during song to establish

138 correlation coefficients for matching and non-matching syllables. For matching syllables

139 the mean correlation coefficient and STD was 0.75±0.23 (n=75) and for comparisons of

140 different syllables of 0.24±0.19 (n=75). The criterion of 0.8 for identification of EMG

141 patterns that match specific syllables therefore constitutes a conservative approach toward

142 identification of nocturnal EMG patterns. No false positive segments were identified with

143 this approach, although some matching EMG segments may have been discarded with this

144 high threshold. Visual inspection of > 500 identified cases was used to further confirm the

145 selectivity of the automated search approach and the choice of 0.8 as threshold correlation

146 value.

147   To quantitatively assess night-time EMG sequences for syllable sequence, we

148   identified the one syllable which occurred most frequently during SLA for each individual.

149   Then we quantified the highest correlation score for all other syllables around the

150   identified syllable. To account for timing differences between song and SLA, we searched

151   within 640 ms near the time point of the syllable as expected from day-time song motifs.

152   From these data we compiled histograms of correlation scores for each individual to assess

153   syllable sequences that occur during SLA.

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157   **Results**

158   Adult male zebra finches produce a stereotyped song sequence that is composed of a

159   repeated series of acoustically distinct syllables, called motif (Zann, 1996; Franz & Goller, 2002;

160   Williams, 2008; Wood et al., 2013). Each song syllable is generated by a stereotyped and

161   characteristic respiratory pulse, which is coordinated with specific activation patterns of

162   syringeal muscles (Fig. 1A,B) (Vicario, 1991a; Goller & Cooper, 2004; Méndez et al., 2010).

163   After recording subsyringeal air sac pressure and electromyograms (EMG) from syringeal

164   muscles in adult male zebra finches during song, we then monitored physiological activity during

165   the night. Syringeal muscles showed spontaneous song-like activation (SLA) during the night.

166   Using a search algorithm based on template matching of the EMG patterns for song syllables, we

167   scanned night-time files for activity. The algorithm showed sufficient distinctive power that

168   permitted reliable identification of EMG patterns from different syllables (see Methods) during

169   day-time song and night-time activity. Even with stringent criteria for the search algorithm, SLA

170   in the syrinx was found remarkably frequently throughout the night, with a range of 110 to 2370

171   (7 birds, over a total of 350 hrs of night-time recording) syllable-like occurrences spread

172   throughout the night-time period (Fig. 2A). However, while SLA occurred in the syrinx, the

173   respiratory system was not simultaneously activated in a song-like fashion and, therefore, no

174   sound was produced. Respiratory rate during syringeal SLA (1.59 ± 0.46 Hz; n = 179; 6 birds)

175   was indistinguishable from normal breathing at night (1.53 ± 0.36 Hz; n=177; 6 birds). Exhalation

176   amplitude and duration were not song-like during SLA. Amplitude expressed as normalized

177   relative voltage output during song was 8 ± 0.23 (n=83 from 5 birds) whereas amplitude during

178   syringeal SLA was 0.9 ± 0.04, n=100 from 5 birds. Additionally, the duration of expiratory

179   pressure pulses during syringeal SLA was 0.61 ± 0.33 s compared to 0.13 ± 0.07 s during song).

180   The prolonged duration of expiration compared to awake breathing indicates that the birds were

181   asleep (Fig. 2B). Of all the SLA events, we only found a different, more song-like respiratory

182   pattern once in one bird, but the pressure was still not sufficiently elevated to result in phonation

183   (Fig. 2B). Although it is unclear whether or not night-time SLA specifically in the syrinx serves

184   a function in maintaining the peripheral or central motor mechanisms, it provides a unique and

185   likely complete record of song-related motor activity in the brain.

186   Although complete EMG patterns of the entire motif were occasionally replayed at night

187   (Fig. 1), most of the EMG activity was not a stereotyped repetition of activity during day-time

188   song. To quantify this, we used mean EMG patterns of individual syllables as a template to scan

189   the night-time files for SLA occurrence and then determined how much of the full song motif

190   was replayed. We chose data from five birds with the most distinctive EMG signals for different

191   syllables. First, we used a subset of SLA occurrences to visually categorize 457 SLA events into

192   one of seven categories of activity (Fig. 3). The categories describe the degree of completeness

193   of the motif and capture deviations in timing from a typical song motif. Of these seven

194   categories, on average over 50% of SLA occurrences fell into the single syllable or partial

195   syllables categories. In contrast, replays of complete motifs occurred in only 7% of the total

196   instances of SLA (Fig. 3D, Table S2).

197   Consistent with previous work (Vicario, 1991a; Franz & Goller, 2002), motor gestures of

198   song production in adult male zebra finches were highly stereotyped (Fig. 4A,B). In contrast,

199   night-time SLA was extremely variable when compared to stereotyped EMG activity of the song

200   motif during day-time song (Fig. 4C). Motor replay consisted of single or multiple syllables

201   without a discernible pattern for which particular syllable in the motif preferentially occurred

202   during SLA. The timing between syllable EMG patterns was also more variable during night-

203   time activity when compared to timing during song. The mean duration between syllables during

204   night-time SLA was longer by 3 ms compared to day-time song and was generally more variable

205   (Fig. 3E).

206   In addition to the variable timing and inconsistent ordering of syllables during SLA, other

207   phenomena occurred in SLA that were never seen in stereotyped song production. EMG patterns

208   of individual syllables showed deviations from the stereotyped pattern of day-time song.

209   Incomplete syllables occurred, with either the first or the last half of the activity pattern missing

210   or only present at a lower EMG amplitude. In other instances, parts of a typical EMG pattern for

211   a syllable were omitted with the rest of the syllable pattern shifted in time, while the other

212   muscle performed the standard song like pattern (Fig. 3C). This caused a mismatch in the SLA

213   pattern between the two syringeal sound generators.

214   Second and in addition to analyzing this subset, we quantified SLA structure for the

215   entire data set. For each bird, we chose SLA occurrences of the most frequently generated

216 syllable and compared the EMG activity near this syllable to all other templates, allowing for

217 different timing. If a full motif was executed, the other cross correlation coefficients should be

218 near our cutoff threshold of 0.8. Any deviations from a full motif will yield low correlation

219 coefficients for the other syllables. The data show clearly for all birds that the full motif was

220 rarely produced (Fig. 4D), as correlation coefficients for other syllables rarely exceed the 0.8

221 cutoff criterion. Furthermore, activation patterns for different syllables occurred at very different

222 rates, because correlation coefficients for some syllables remained much lower than those for

223 others. Whereas the mean correlation coefficients for the target syllables ranged from 0.82-0.89,

224 those for the other syllables ranged from 0.39-0.82. Finally, this analysis reveals differences

225 between individuals in respect to the syllable composition of SLA sequences (Fig. 4D).

226

227 **Discussion**

228 Here we have shown that night-time SLA does not only occur in the forebrain circuitry (HVC

229 and RA - Dave & Margoliash, 2000; Hahnloser, Kozhevnikov & Fee, 2002; 2006; Area X -

230 Yanagihara & Hessler, 2012), but that this activity also is relayed to the syringeal muscles.

231 Whereas during song production RA initiates activation of both respiratory pre-motor circuits

232 and the syringeal motor neurons (nXIIts) (e.g., Vicario, 1991b; Roberts et al., 2008; Schmidt,

233 McLean & Goller, 2012), during SLA only activation of the syringeal motor system occurs. The

234 specific mechanism for disengaging respiration from the song motor sequence is not known, but

235 inhibition at the level of the respiratory pre-motor nuclei may prevent the song-like activation of

236 the respiratory system and, thus, sound generation.

237   The functional significance of activating the syringeal muscles during night-time motor

238 replay is unclear. Because song generation involves coordinated activity of respiratory, vocal

239   organ and upper vocal tract motor systems, the activation of only one of them during SLA is

240   unlikely to provide useful peripheral feedback information for consolidation of the central song

241   motor program. The activation of the whole motor circuit including the syringeal motor nucleus

242   (nXIIts) may be necessary for providing benefits to the maintenance of the motor program.

243   Alternatively, the activation of the syrinx may not be serving any function for song program

244   consolidation, but, unlike respiration, may not have been selected against as it produces no

245   externally visible movement and is likely not very energetically costly (Oberweger & Goller,

246   2001; Franz & Goller, 2003). Whether or not it may provide stimuli needed for the homeostatic

247   maintenance of the superfast syringeal muscles remains unclear (Elemans et al., 2008; Uchida et

248   al., 2010).

249   Nevertheless, the feed through of central activity to the syrinx provides the first detailed

250   insight into the frequency of occurrence and specific features of motor replay of song-like

251   activity in the brain. Such detailed information is not available for any complex behavior and,

252   thus, enables us to characterize spontaneous motor activity for the first time. SLA occurred

253   throughout the night period and very frequently. Replayed motor gestures for individual syllables

254   are not consistently ordered into the correct motif sequence, are often incomplete, and even

255   coordination of the left and right motor pathways to the two sound generators of the syrinx is not

256   consistently present.

257   These findings are largely consistent with spontaneous RA activity during sleep (Dave &

258   Margoliash, 2000). Individual neurons in RA spike at specific syllable segments and are silent

259   during the remaining song components. A full assessment of song-like activity during sleep is

260   therefore not possible from these neuronal data. Whereas the spike patterns of spontaneous

261   activity during sleep can be matched to activity during song performance, the absence of spikes

262   and non-matching bursts are difficult to interpret (Dave & Margoliash, 2000; Shank &

263   Margoliash, 2009; Rauske et al., 2010). Syringeal activation therefore provides more information

264   about which song elements are replayed and permits the interpretation that sleep activation is not

265   replay of the stereotyped motor program for song. This conclusion therefore differs from other

266   systems where night-time activation is thought to replay previously experienced sequences fairly

267   faithfully, albeit with different tempos, to consolidate newly acquired memories (e.g., Wilson &

268   McNaughton, 1994). In contrast to these findings, the motor replay patterns in adult zebra

269   finches suggest that night-time activity constitutes a highly variable motor activation that may be

270   similar to plasticity experienced during vocal learning (Tchernichovski et al., 2001;

271   Derégnaucourt et al., 2005; Shank & Margoliash, 2009; Ölveczky et al., 2011).

272   During the sensorimotor period of song development, birds "practice" song production

273   and generate error signals in the auditory feedback that are thought to be used for refining

274   imitation of the acquired song model. Interestingly, SLA in HVC occurs less frequently in young

275   birds during the sensorimotor phase than in adult birds (Crandall et al., 2007). In adult birds,

276   however, song production is highly stereotyped, and variation in tempo and acoustic parameters

277   (e.g., frequency) is very small (1-5%; e.g., Franz & Goller, 2002; Cooper & Goller, 2006;

278   Crandall et al., 2007; Williams, 2008; Méndez et al., 2010; Wood et al., 2013). Frequent SLA

279   and its high variability relative to the stereotyped motif of day-time song could be related to the

280   maintenance of the motor program.

281   A possible mechanism for how variable SLA enhances the stability of the song motor

282   program is the generation of internal error signals that contribute to motor stability. An efference

283   copy of HVC activation is thought to be involved in auditory feedback controlled song learning

284   (e.g., Troyer & Doupe, 2000; Prather et al., 2008; Mooney, 2009; Bolhuis, Okanoya & Scharff,

285   2010; Brainard & Doupe, 2013; Fee, 2014). In adult birds this efference copy could predict

286   motor output, which could internally be compared to the motor representation of stereotyped

287   song. This internal error generation may counteract the decay in motor memory that is predicted

288   in the absence of error signals (e.g., Kitago et al., 2013; Vaswani & Shadmehr, 2013; Brennan &

289   Smith, 2015) and may facilitate the incorporation of new neurons into the song circuit (e.g.,

290   Alvarez-Buylla, Thelen & Nottebohm, 1988; Nottebohm, 2008; Pytte et al.2012) without

291   jeopardizing the stereotypy of day-time singing. This proposed model makes clear predictions

292   that are testable. If this proposed mechanism is at work in the maintenance of the motor program

293   for highly stereotyped song production, it likely constitutes a more general mechanism for how

294   complex motor programs that are generated in a distributed network of brain areas may be

295   maintained over long periods of time (Criscimagna-Hemminger & Shadmehr, 2008; Vaswani &

296   Shadmehr, 2013).

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462   **Ethics:** All procedures and experiments were performed in accordance with protocols approved
463   by the Institutional Animal Care and Use Committee (IACUC) at the University of Utah.
464
465   **Author contributions:** BY and FG designed the study and conducted experiments, all authors
466   contributed to data analysis, all authors wrote and edited the manuscript.
467

468   **Acknowledgements:** This research was funded by the NIH. E.M.A was supported by a Premio
469   Fundación Bunge y Born postdoctoral fellowship.

470
471   **Competing Interests:** The authors have no competing interests.

473 **Figure legends:**

474

475 **Fig. 1**. Song-like activation of syringeal muscles occurs at night without sound generation. (A)

476 Day-time song (shown spectrographically) is accompanied by a stereotyped air sac pressure

477 pattern (P, horizontal line illustrates ambient pressure, relative units) and EMG activity (vTB$_L$

478 and dS$_L$ are left ventral tracheobronchial and left dorsal syringeal muscles). Colored boxes

479 outline individual syllables, and the corresponding colored bars indicate the EMG activity

480 associated with that syllable. (B) An example of SLA during the night showing activation of

481 syringeal muscles without concurrent song-like air sac pressure patterns. EMG patterns of

482 individual syllables are identified by color bars as in A. SLA includes multiple syllable repeats

483 with one syllable missing. (C) Examples of syllable-specific EMG patterns of the motif of one

484 bird (templates) with a complete motif sequence from night-time activity for comparison as

485 identified in the automated search procedure.

486

487

488 **Fig. 2.** (A). SLA occurred throughout the night. Specific syllables are identified by color and are

489 replayed with varying frequency during the night. (B). Respiration during night-time SLA

490 (green data point) was normal quiet respiration during sleep and differed in amplitude and

491 duration from daytime quiet respiration (blue) as well as from day-time singing (red). In only one

492 individual night-time SLA occurred occasionally with simultaneously song-like duration and

493 increased amplitude of expiratory pulses (yellow data point). Values are means ± 1 s.d. (n=250;

494 except for left green data point n=3).

495

496 **Fig. 3.** SLA showed lack of left-right coordination. (A) Respiration during song with colored

497 boxes outlining individual syllables. (B) EMG activity pattern in the indicated syringeal muscle

498 during song. The upward trace shows a rectified (time constant 0.1 ms) and integrated (5 ms)

499 EMG signal and the downward trace is a rectified raw signal for comparison. EMG patterns

500 which correspond to a certain syllable are underlined with the respective color. (C) An example

501 of SLA with altered syllable sequence (red arrow indicates missing syllable). (D) Occurrence of

502 SLA patterns assigned to different categories for illustration of variability. Less than 7% of all

503 occurrences were complete song motifs. (E) The timing of syllable like patterns during SLA was

504   also more variable (red) than inter-syllable intervals during song (black). To quantify

505   intersyllable intervals, we arbitrarily set a threshold of 200 ms on either side of a SLA pattern for

506   it to be considered independent of another. Large negative intervals (>20 ms) in the SLA

507   histogram indicate that the beginning of an EMG pattern for a syllable was missing with the last

508   half shifted back. Measurements were taken as if the entire syllable pattern were present.

509

510   **Fig. 4.** Night-time SLA is highly variable compared to activation during song. (A-C) Overlaid

511   traces (different colors) of 15 song motifs illustrate the high stereotypy of song production (A.,

512   subsyringeal air sac pressure, B, rectified and integrated EMG of left and right ventral syringeal

513   muscles). During SLA in contrast, EMG patterns in the same muscles are much more variable

514   (20 different occurrences overlaid). (D) Distribution of correlation coefficients for EMG activity

515   around the most frequently produced syllable (black bars) for each bird. The low scores for

516   activity around the target syllable indicate that the full sequence is rarely produced and different

517   syllable patterns are generated at different frequencies. For example, in the first bird syllable 3 is

518   the most frequently produced syllable, and the correlation scores for syllables 4 and 5 show that

519   they are less frequently produced than 1 and 2.

520

522   **Supplementary material**

523
524

525   Fig. S1

526
527   Each syringeal half is controlled by at least six muscles. The four largest muscles are indicated
528   (vS, ventral syringeal, vTB, ventral tracheobronchial, dTB, dorsal tracheobronchial, dS, dorsal
529   syringeal; modified from *23*). Recordings were made from 3 of these on both sides (see Table
530   S1).

531

532   Tables S1-S#

533   Table S1: Recorded muscles for each bird.

Bird	BR192	G81	G74	G51	P98	P106	P145	P144	P188	P181
Muscles	dS_L*, vTB_L	dS_L, vTB_L	vTB_L, vTB_R	vTB_L, vTB_R	vS_L, vS_R	vTB_L, vS_R	dS_R, vTB_R	dS_R, vTB_R	vTB_L, vTB_R	vS_L, vS_R

534   *L and R indicate left or right muscle.

535   Table S2: Average occurrence of each SLA type with standard error.

Category	1 Syllable	Syllable + unknown	Two or more syllables	Two or more + unknown	Complete motifs	Partial syllables	Incorrect timing
Percent occurrence	20±6.6%	12±4.2%	15±4.3%	5.5±2.2%	6.1±2.2%	33±7.4%	8.1±2.0%

536

537

Figure 1

EMG patterns during song and sleep

Song-like activation of syringeal muscles occurs at night without sound generation. (A) Day-time song (shown spectrographically) is accompanied by a stereotyped air sac pressure pattern (P, horizontal line illustrates ambient pressure, relative units) and EMG activity (vTB_L and dS_L are left ventral tracheobronchial and left dorsal syringeal muscles). Colored boxes outline individual syllables, and the corresponding colored bars indicate the EMG activity associated with that syllable. (B) An example of SLA during the night showing activation of syringeal muscles without concurrent song-like air sac pressure patterns. EMG patterns of individual syllables are identified by color bars as in A. SLA includes multiple syllable repeats with one syllable missing. (C) Examples of syllable-specific EMG patterns of the motif of one bird (templates) with a complete motif sequence from night-time activity for comparison as identified in the automated search procedure.

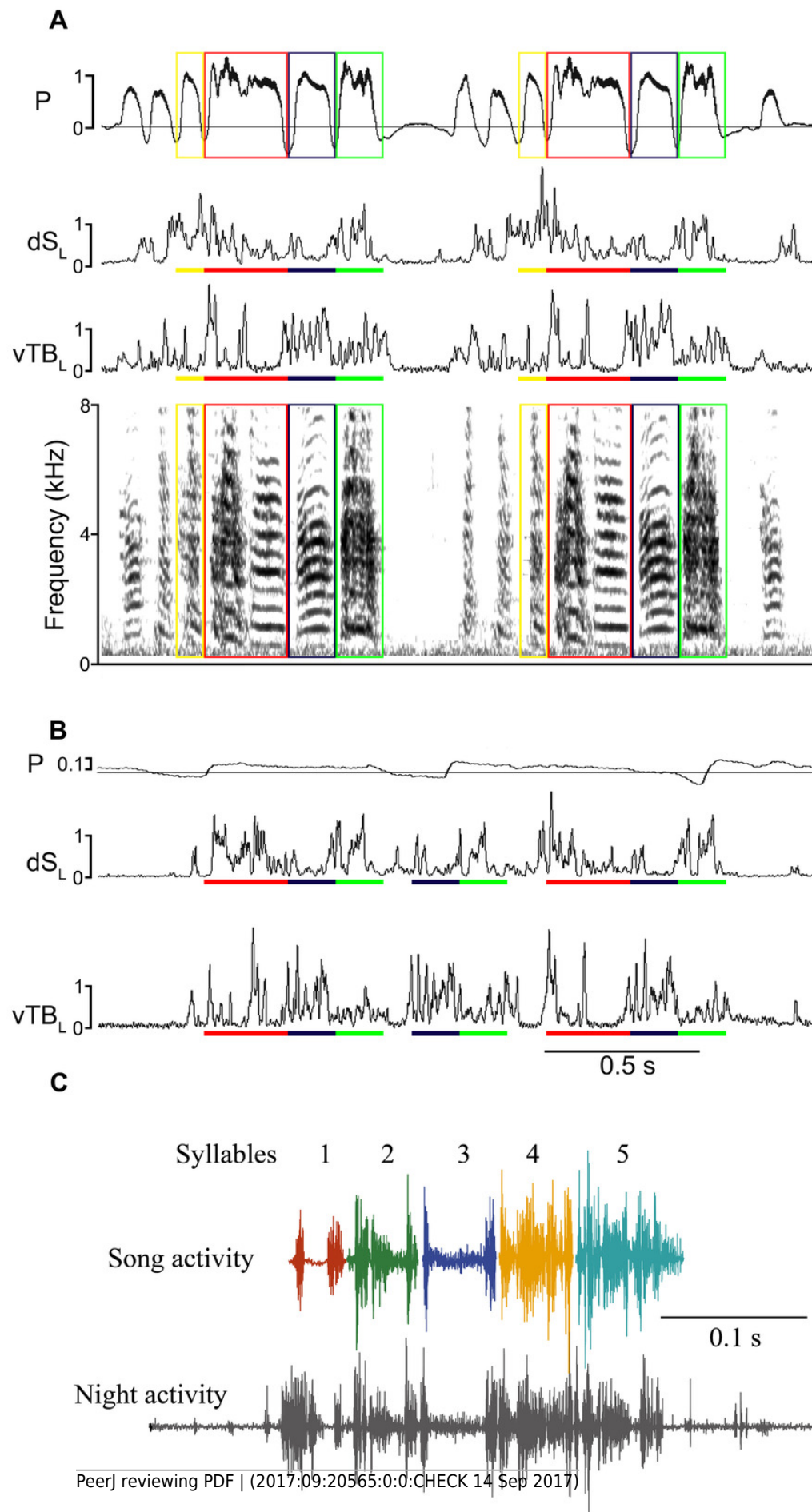
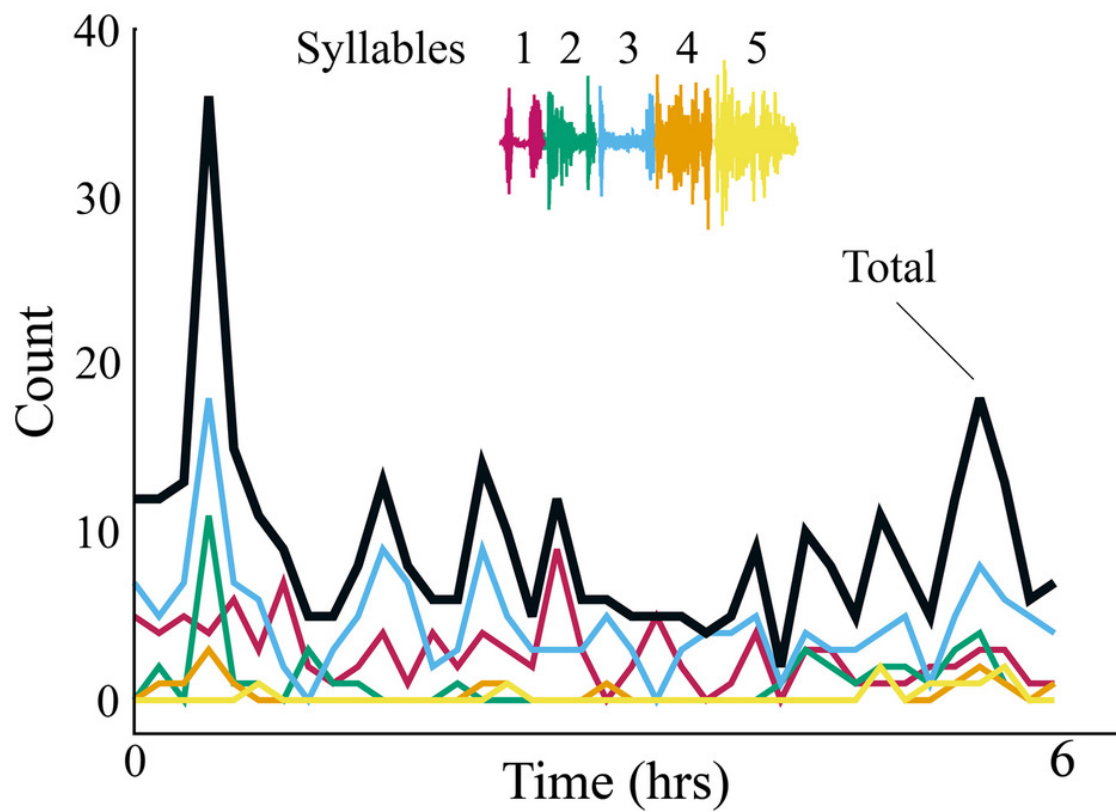



Figure 2

Night-time activation and lack of respiratory activation

(A). SLA occurred throughout the night. Specific syllables are identified by color and are replayed with varying frequency during the night. (B). Respiration during night-time SLA (green data point) was normal quiet respiration during sleep and differed in amplitude and duration from daytime quiet respiration (blue) as well as from day-time singing (red). In only one individual night-time SLA occurred occasionally with simultaneously song-like duration and increased amplitude of expiratory pulses (yellow data point). Values are means ± 1 s.d. (n=250; except for left green data point n=3).

A

B

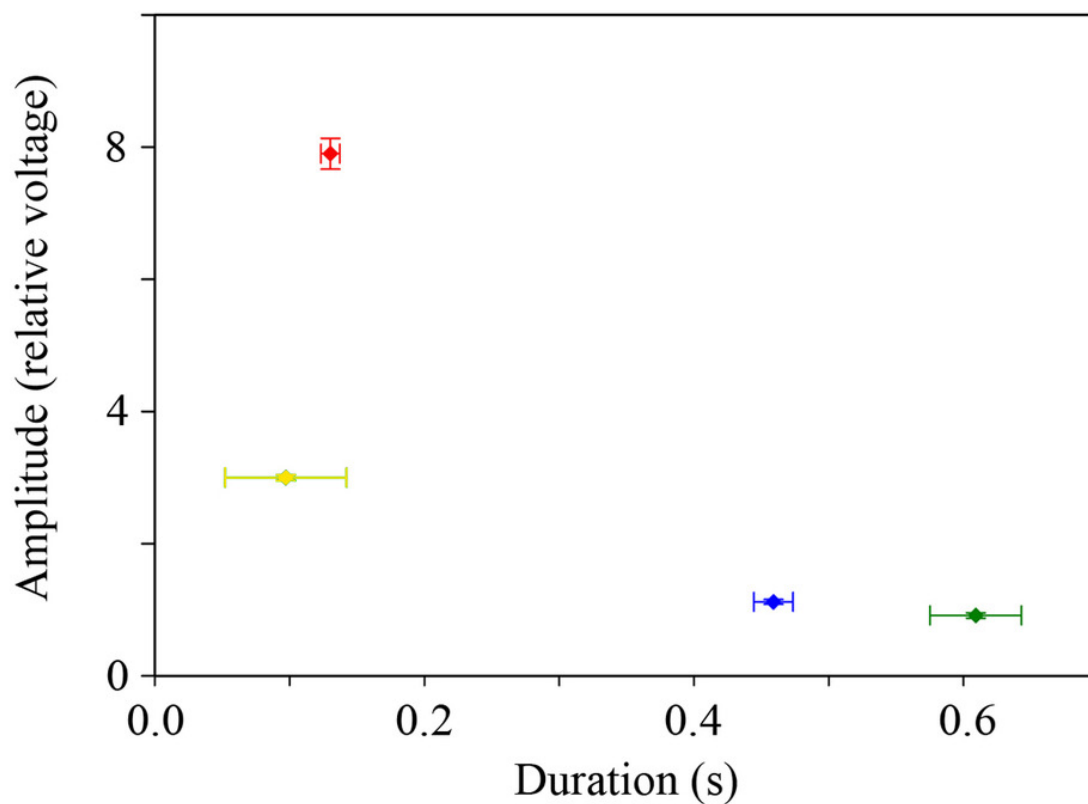



Figure 3

Syllable sequencing during night-time activation

SLA showed lack of left-right coordination. (A) Respiration during song with colored boxes outlining individual syllables. (B) EMG activity pattern in the indicated syringeal muscle during song. The upward trace shows a rectified (time constant 0.1 ms) and integrated (5 ms) EMG signal and the downward trace is a rectified raw signal for comparison. EMG patterns which correspond to a certain syllable are underlined with the respective color. (C) An example of SLA with altered syllable sequence (red arrow indicates missing syllable). (D) Occurrence of SLA patterns assigned to different categories for illustration of variability. Less than 7% of all occurrences were complete song motifs. (E) The timing of syllable like patterns during SLA was also more variable (red) than inter-syllable intervals during song (black). To quantify intersyllable intervals, we arbitrarily set a threshold of 200 ms on either side of a SLA pattern for it to be considered independent of another. Large negative intervals (>20 ms) in the SLA histogram indicate that the beginning of an EMG pattern for a syllable was missing with the last half shifted back. Measurements were taken as if the entire syllable pattern were present.

A

B

vS$_L$

vS$_R$

C

0.2 s

D

E

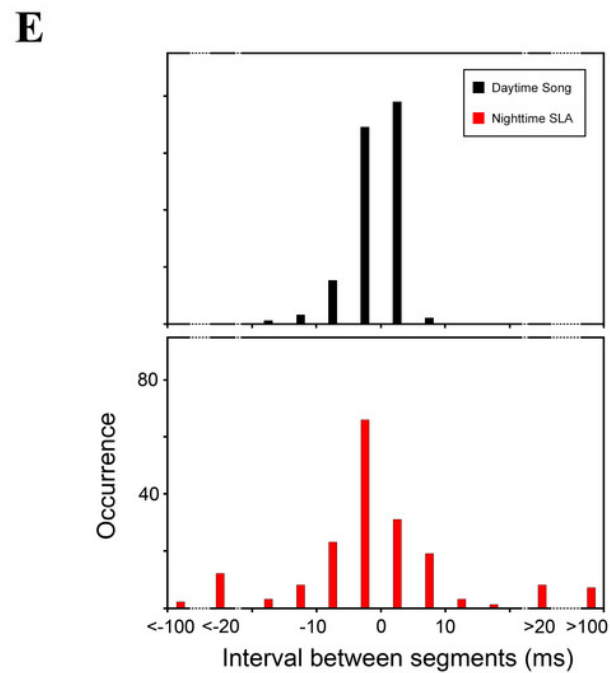
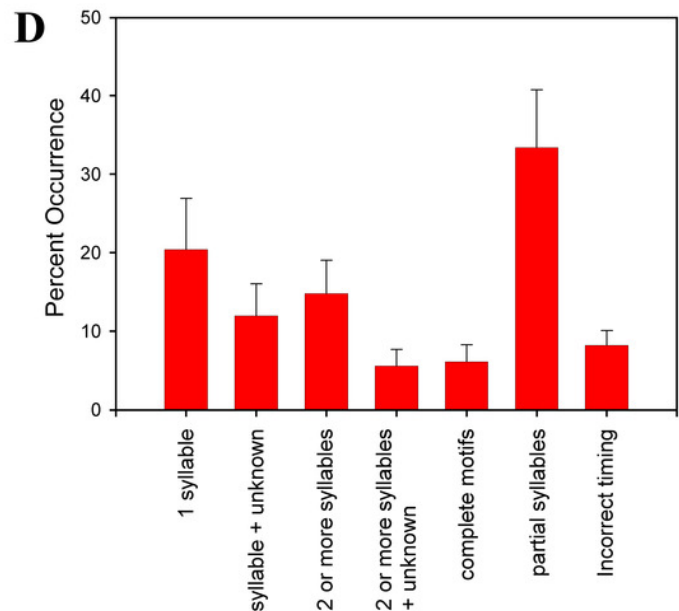
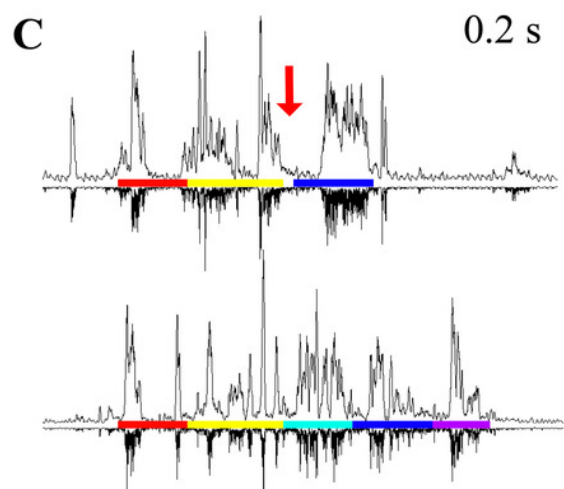
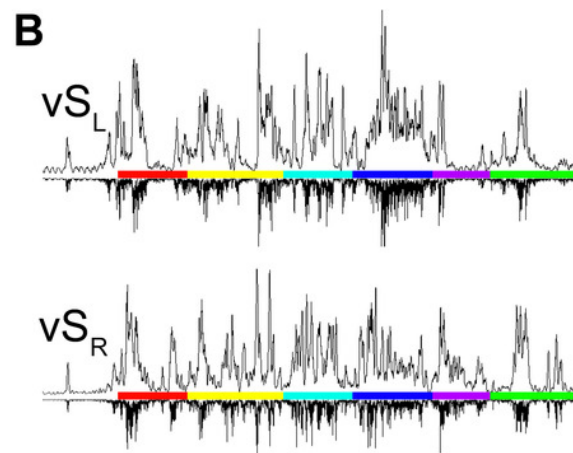
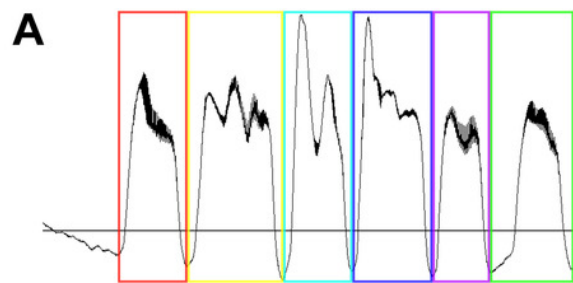



Figure 4

High variability of sequence during night-time activation

Night-time SLA is highly variable compared to activation during song. (A-C) Overlaid traces (different colors) of 15 song motifs illustrate the high stereotypy of song production (A., subsyringeal air sac pressure, B, rectified and integrated EMG of left and right ventral syringeal muscles). During SLA in contrast, EMG patterns in the same muscles are much more variable (20 different occurrences overlaid). (D) Distribution of correlation coefficients for EMG activity around the most frequently produced syllable (black bars) for each bird. The low scores for activity around the target syllable indicate that the full sequence is rarely produced and different syllable patterns are generated at different frequencies. For example, in the first bird syllable 3 is the most frequently produced syllable, and the correlation scores for syllables 4 and 5 show that they are less frequently produced than 1 and 2.