



# Song-like activation of syringeal and respiratory muscles during sleep in canaries

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## Abstract

Sleep replay activity involves the reactivation of brain structures with patterns similar to those observed during waking behavior. In this study, we demonstrate that adult male canaries exhibit spontaneous, song-like peripheral reactivation during night sleep. Our findings include: (1) the presence of activity in respiratory muscles, leading to song-like air sac pressure patterns of low amplitude, (2) the simultaneous occurrence of respiratory replay events and reactivation of syringeal muscles, and (3) the reactivation of syringeal muscles without concurrent respiratory system activity. This song-like reactivation of peripheral motor systems enables the identification of specific motor patterns, with replay events preserving individual morphological and temporal properties. The activation of peripheral motor systems in songbirds and the differences in activation patterns between species give unique insights into the fictive behavioral output of activation of a complex learned motor behavior during sleep, shedding light on the neural control mechanisms and potential functions.

**Keywords** Motor replays · Sleep · Oscines · Birdsong · Respiratory muscles

## Introduction

In humans and nonhuman animals, diverse brain areas (hippocampus, cortical areas) display neural activation during sleep (for review e.g., Chen and Wilson 2023). While there is ample evidence that this activity plays an important role in memory consolidation (for review e.g., Klinzing et al. 2019; Brodt et al. 2023; Goto and Hayashi 2023), its specific role in establishing and consolidating motor mechanisms is somewhat less clear (e.g., Ramanathan et al. 2015; Maier et

al. 2017; van den Berg et al. 2019; Xu et al. 2019; Cheng et al. 2021). Activation may resemble replay, preplay or other variations on normal daytime activity (e.g., Dragoi and Tonegawa 2011; Liu and Watson 2020; Rubin et al. 2022). While most work has focused on mammals, birds also display reactivation of motor patterns during sleep (Dave and Margoliash 2000; Hahnloser et al. 2002; Young et al. 2017; Elmaleh et al. 2021). Specifically, motor behavior associated with singing provides an interesting system for comparison. Like for hippocampal control of spatial behavior in rodents (e.g., Moser et al. 2015; Robinson et al. 2020), neural mechanisms for song production have been elucidated (for review e.g., Mooney 2020; Murphy et al. 2020; Nieder and Mooney 2020), such that the emergence and expression of neural activation patterns during sleep can be compared to daytime singing. Additionally, in some bird taxa, species-specific song production is an imitative, learned motor behavior (in oscine songbirds, parrots and hummingbirds), while in others it develops innately (e.g., Jarvis 2019; Arato and Fitch 2021; Vernes et al. 2021).

Song-like night-time activation was initially detected in areas of the cortical song control circuit of oscine songbirds, but these recordings did not allow determination of the specific song components that were ‘replayed’ (Dave and Margoliash 2000; Hahnloser et al. 2002). The discovery

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that song-like night-time activation can be monitored in the musculature controlling the avian vocal organ, the syrinx, revealed that zebra finches (*Taeniopygia guttata*) do not 'rehearse' the stereotyped song motor patterns of the daytime behavior but 'replayed' night-time activity displays high variability (Young et al. 2017). Because activation patterns of syringeal muscles show high specificity for different song elements, precise information about which song elements were replayed during sleep could be attained. Song-like activity rarely is a replay of the full song motif but displays uncoordinated activity of the left and right sound generators, incorrect syllable sequence and song motif fragments (Young et al. 2017; Elmaleh et al. 2021, 2023). Interestingly, song-like activation of syringeal muscles during sleep also occurs in non-vocal learning suboscines (Döppler et al. 2021). While vocal imitation learning requires elaborate cortical and basal ganglia circuitry, song in non-vocal learners is thought to be controlled by the midbrain dorso-medial nucleus of the intercollicular complex (DM), and night-time activation in these birds may not involve cortical motor areas.

Song-like activation during sleep is therefore ideally suited for probing the neural mechanisms of song motor control and its evolution toward increased cortical control of development and execution. In the zebra finch, depriving the cortical song control circuit (HVC, proper name and RA, robust arcopallial nucleus) of thalamic input (lesions of the uvaeform nucleus or Nif, nucleus interfaccialis) did not prevent song-like activation of neurons in RA, and activation patterns suggest that such input is not needed for syllable-to-syllable progression of premotor activity (Elmaleh et al. 2021). Coordination between the left and right cortical song control circuits is thought to be achieved by thalamic input. It is therefore consistent with the finding that night-time song-like activity shows a lack of coordination (Elmaleh et al. 2023). These findings are consistent with the observations on the involvement of peripheral motor systems in night-time song-like activation. While syringeal muscles are activated in song-like patterns, the respiratory system is not simultaneously activated. During song, the respiratory system generates distinct, stereotyped pressure pulses, which correspond to song syllables, and replenish air during the pauses in between song syllables via deep short inspirations, mini-breaths. During sleep in zebra finches, song-like activity does not engage the respiratory system, such that breathing continues as slow, shallow breaths, typical of sleep (Young et al. 2017).

These findings in the zebra finch provide important detail in a species whose song motor control has been studied most extensively. However, to fully understand neural control of song production and the possible functions of how song-like activity during sleep ties into development and maintenance

of the song motor program, a more comparative perspective is needed. Here we investigate another vocal imitation learning oscine species, the canary (*Serinus canaria*). Canaries exhibit complex songs that can last > 30 s and consist of sequences with whistles and trills of different trill rates. Trills are composed of syllables, the elementary sound units which are repeated. Syllables are stereotyped sounds that can contain one or more song elements (sounds separated by silent gaps). Songs are generated with rapid and complex respiratory activity, where trill rates up to 30. s<sup>-1</sup> are generated such that individual expiratory pulses generate each syllable of the trill, while pauses in between syllables are used for short, deep mini-breaths. Higher trill rates are generated during sustained expiratory pulses (Hartley and Suthers 1989; Suthers et al. 2004). Activity of syringeal muscles regulates airflow during this rapid switching between respiratory phases and controls acoustic features, such as amplitude and frequency (Goller and Suthers 1996a, b). Unlike zebra finches, adult canaries as well as other species can modify their songs between breeding seasons and display different sensorimotor gating of feedback information to the song control circuitry of the forebrain (e.g., Prather et al. 2008; Lehongre and Del Negro 2009).

These differences in song organization, plasticity, and maintenance make canaries an ideal species for expanding our knowledge of nighttime song replay. In the present work, we first demonstrate that song-like night-time activity also occurs in canaries. However, in addition to song-like activation of syringeal muscles, canaries sometimes also activate expiratory muscles. Crucially, each canary syllable is generated by a precise air sac pressure pattern with a morphology and time scale very different from normal breathing (Trevisan et al. 2006; Alonso et al. 2014; Fainstein et al. 2021). This allowed us to unambiguously show that replay activity can be detected in both expiratory muscles and air sac pressure during night-time activation. Understanding whether activation patterns differ between species can shed light on potential differences in song sensorimotor control and further our knowledge of nighttime neural motor activity and its potential functions. Overall, the complete activation of the respiratory system and the vocal organ during night sleep paves the way for a quantitative and interpretable study of replay activity of a complex learnt motor behavior (Döppler et al. 2024).

## Materials and methods

### Experimental procedures

Experiments were conducted on five male canaries (*Serinus canaria*), according to the regulation of the animal care

committee of the University of Buenos Aires. Birds were acquired from a local breeder and were more than one year old (Roller canary strain). Canaries were housed individually in 27 cm x 23 cm x 20 cm wire cages inside an acoustic chamber. The chamber's door was usually opened, and the subjects had intermittent (but daily) auditory and visual contact with other canaries. They were fed with food and water *ad libitum*. The photoperiod was set from 6:00AM to 8:00 PM and was controlled by an automatic timer. Measurements were done between October and March (South American spring and summer) while males were in breeding condition. The detail of the dates in which each experiment was conducted can be seen in Supplementary Table S1.

Canaries were accustomed to the procedures at least two weeks before the surgery by attaching a backpack to the bird (see Supplementary Table S1). The backpack is custom-built and consists of a Velcro tab on the back that is secured on the bird with elastic bands around the base of each wing. Birds were topically treated with 20 mg testosterone gel (1%; Androlone, Laboratorios BETA, Argentina), equivalent to 0.2 mg of testosterone, during the five days prior to the surgery to stimulate song during the day (Alliende et al. 2010). During this stage, and before any surgical procedure was performed, song was recorded using a directional microphone (Venetian HT-81 A) and audio amplifier (Behringer MIC100). No clear changes in song behavior were observed during the time in which the experiments were performed (see Supplementary Methods and Supplementary Fig. S1).

In each bird, we recorded air sac pressure and electromyographic (EMG) activity in the expiratory abdominal muscle sheet with sound. In two of these birds, air sac pressure and EMG activity of the left or right syringealis ventralis (vS) muscle was also recorded (see Supplementary Table S2).

Surgery was performed under general anesthesia using isoflurane. During surgery, a flexible silicone cannula (AM-systems Silicon Tubing 0.030" x 0.065" x 0.0177" Catalog No. 807000) was inserted right below the last rib, 5 mm into the anterior thoracic air sac. The cannula was sutured to the rib cage, and the incision site was sealed with tissue cement (Vetbond; 3 M Animal Care Products, St. Paul, MN, USA). The free end of the cannula was attached to a piezoresistive pressure transducer (FHM-02 PGR, Fujikura, Tokyo, Japan), positioned in the backpack. Differential EMG activity was recorded with a pair of fine (<0.01 cm diameter), custom-built, silver-plated copper wires (44TDQ, Phoenix Wire Inc., VT, USA). To record from expiratory abdominal muscles, the bare wire tips (0.2 cm length) of the electrodes were aligned in parallel and inserted into the thin muscle sheet and fixed in place by tissue adhesive (Vetbond) applied to the muscle surface. The syrinx was accessed by

a skin incision in the furcula area and opening of the interclavicular air sac membrane. The tips of the electrodes were inserted into the syringeal muscle and adhered to the surface with tissue adhesive (Vetbond). For both EMG measurements, insulated leads were routed under the skin to the birds' back and connected to the backpack from which stronger wires led to the recording unit.

Following surgery, all conditioned signals were acquired using a National Instruments acquisition board (NIDAQ-USB-6212 or NIDAQ-USB-6259). Recordings were controlled using a custom MATLAB script at 44.15 kHz sample rate. Pressure signal was connected, before digitization, to a custom-built variable gain analog amplifier (300 x maximum gain). EMG signals were processed with a 150 Hz high-pass RC filter and with an analog differential amplifier (225 x), both printed on the backpack. All custom-built analog devices were powered by external 12-V batteries to avoid line noise. A Butterworth digital filter (5th order, 300 Hz cutoff) is applied forward and backward to song pressure recordings (SciPy for Python). Signals were recorded continuously during the night, and daytime recordings were recorded continuously or triggered by sound level. During night-time recordings, we assessed the birds' state using an infrared camera (Sony HDR-SR7) to verify that their eyes were closed, no movements occurred, and birds had a sleep-like posture (Ungurean et al. 2020).

## Data analysis

### Automatic clustering of song air sac pressure patterns

Song air sac pressure recordings were segmented into syllable-related fragments (referred to as pressure patterns) by cutting the signal between consecutive subatmospheric minima. In that way, we obtained the air sac pressure fluctuation related to each syllable (the minimum sound unit that is repeated). A cubic spline interpolation was then performed on each air sac pressure pattern time series (SciPy for Python). The spline was evaluated at a constant time step such that each time series was resampled to have 1,000 points. These time series were then mapped to [-1,1] using the following function:

$$f(p(t)) = 2 \frac{p(t) - \min(p)}{\max(p) - \min(p)} - 1$$

where  $p(t)$  represents the air sac pressure pattern time series, and  $\min(p)$  and  $\max(p)$  are its minimum and maximum values, respectively.

For each bird, all resampled and normalized song pressure patterns were grouped into an  $N_s \times 1000$  matrix, where  $N_s$  is the number of syllables for that bird. A two-dimensional

Uniform Manifold Approximation and Projection (UMAP) was then performed (umap-learn for Python). This process mapped each song pressure pattern into a two-dimensional space, resulting in clearly separated clusters. These clusters were algorithmically found using Hierarchical Density-Based Spatial Clustering of Applications with Noise (HDBSCAN), with a minimum cluster size of 2% of the dataset size (scikit-learn for Python) (Campello et al. 2013).

### Search of vS song-like activity during night sleep

Comparison of spontaneous vS EMG activity to vS EMG activity during song was done using custom written software, written in Python, implementing the following procedures.

First, we selected representative song vS EMG fragments as follows. Using the simultaneous measurement of air sac pressure and muscle activity, we segmented the EMG data into syllable-related fragments (referred to as vS EMG patterns). We computed the envelope of the absolute value of the EMG signal and cut it at specific time points. These time points were determined by identifying the minima of the pressure patterns with subatmospheric values, then locating the next immediate minimum for each identified minimum. These times were used to segment the EMG envelope into parts corresponding to each song syllable. We selected segments of long syllables (> 100 ms) that are usually sung without immediate repetition and, for short syllables (< 100 ms) that are repeated many times, we selected segments containing three successive syllables of the same trill, to capture the morphology and the periodicity of the signal. These EMG segments corresponding to song patterns are hereafter called song templates. We selected five templates for each bird (see Supplementary Figure S2).

The envelopes of EMG signals were computed using a first order integration,

$$\frac{dx}{dt} = -\frac{1}{\tau}x + EMG(t),$$

with  $\tau = 0.01s$ . Here  $EMG(t)$  denotes the absolute value of the recorded signal and  $x(t)$  the computed envelope.

Secondly, continuous EMG activity was recorded during both daytime and night sleep and divided into 30 s time series. For each time series, we computed its envelope  $x(t)$ . The aim was to determine, for every envelope, if it contained song-like activity. We computed the Pearson correlation between the envelope and each song template, shifting at 2 ms intervals from 0 s to  $30s - T_{template}$ , where  $T_{template}$  is the duration of the template analyzed. This resulted in a continuous time series for each template, representing its correlation with the EMG envelope. Correlation values for low-amplitude parts of

$x(t)$  were set to zero, with the activity threshold defined as two standard deviations of the mean EMG amplitude during song production (an example is shown in Supplementary Figure S2).

We first analyzed daytime (awake) activity. For each time series  $x(t)$ , we counted the number of high-correlation events, defined as the sum of correlation peaks above a threshold for all the templates (referred to as  $N_{hc}$ ). Results for the birds in which vS EMG was measured are shown in Supplementary Figure S3. Identifying which time series corresponded to song production (since during the day sound was produced) enabled us to sample  $N_{hc}$  distributions for both song and non-song time series. Analyzing night sleep activity, using  $N_{hc}$  as the statistic, we tested whether a nighttime time series exhibited song-like activity (see Supplementary Table S3).

### Statistical analysis

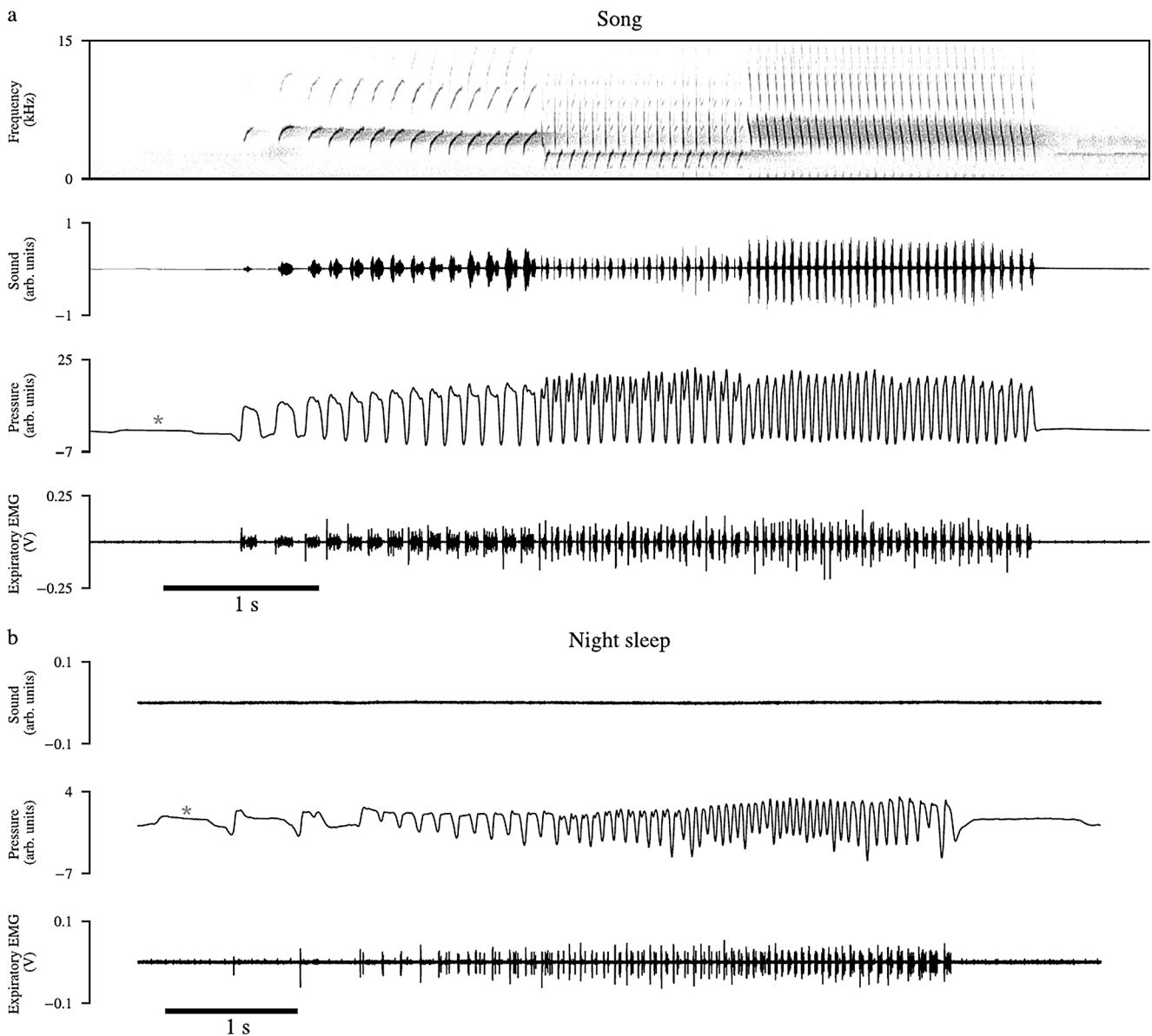
Statistical analyses were performed using Python. Welch's t-test for mean values comparisons were performed using SciPy's Stats module for Python.

## Results

### Replay in the respiratory system

The song of adult canaries consists of the repetition of a set of syllables, grouped into phrases that are combined in variable sequences (Markowitz et al. 2013). Each syllable is generated during the expiratory phase of a characteristic respiratory pulse, produced by the action of expiratory muscles. At syllable repetition rates below  $30. s^{-1}$ , silent gaps in between repeated syllables correspond mini-breaths (Fig. 1a). In sleeping birds at night, we discovered events where the expiratory muscles exhibited spontaneous song-like activity (SLA), which translated into air sac pressure fluctuations (Fig. 1b). These expiratory EMG and pressure patterns share strong similarities in morphology and timing with the activity observed during daytime song production (Fig. 1a), are different from basal respiratory activity (see Supplementary Fig. S4) but are not exact copies of a song observed during the day. The amplitude of expiratory song-like pressure patterns is lower than during day-time song, and no sound is generated. This low-amplitude dynamics was never observed during waking behavior.

The structure of the result section is as follows. We start by performing a statistical test to determine whether a night-sleep pressure fluctuation corresponds to a song-like pressure pattern. Then, we address the questions of what pressure patterns are replayed at night, to what degree rhythmic properties are preserved and how long these events are. In



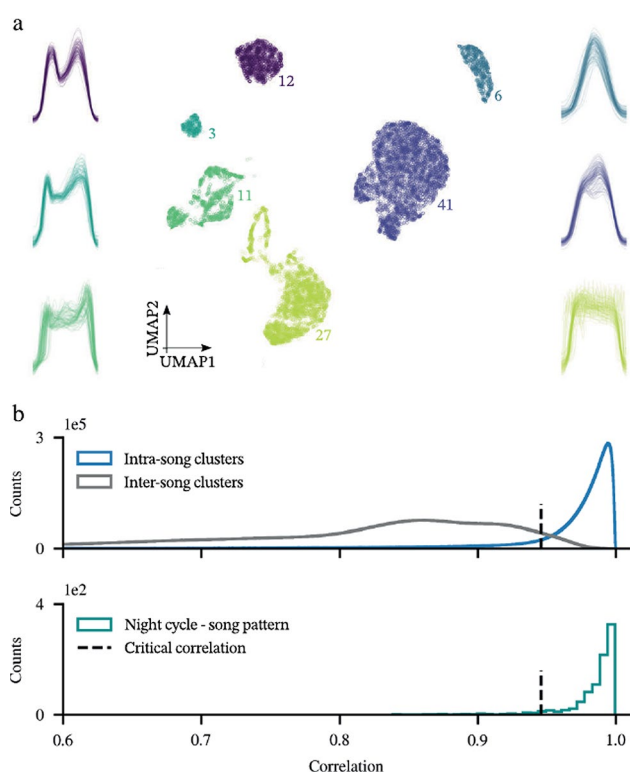
**Fig. 1** **a** Adult canary respiratory activity during song. The first panel shows the spectrogram of the recorded sound, which is also displayed oscillographically in the second panel. Each sound syllable is associated with a specific pressure and expiratory EMG patterns. **b** Spontaneous song-like re-activation during night sleep. The typical normal breathing pressure fluctuation (examples marked with an asterisk) is

interrupted by pressure patterns that exhibit morphological and temporal similarities with song activity. The amplitude of expiratory song-like pressure patterns is lower than during day-time song, so no sound is produced. All pressure signals are normalized so that normal respiration oscillates between  $-1$  and  $1$  (see Supplementary Methods). Recordings correspond to bird 1

the next section we show that canaries exhibit spontaneous song-like re-activation of syringeal muscles, that respiratory replay events are always simultaneous to the reactivation of vocal muscles, but that syringeal SLA can occur without concurrent respiratory SLA. We address how often SLA events occur and assess their distribution during the night. Finally, we compare the amplitude of air sac pressure, expiratory EMG and vS EMG during song and SLA.

We performed an extensive inspection of respiratory activity during the night to search for SLA events.

Throughout most of the night, the birds exhibited normal, slow ( $\sim 1$  Hz) respiratory pressure fluctuations, which are characterized by low amplitude of exhalatory and inhalatory pressure pulses (see Supplementary Fig. S4). We selected events where normal respiration was interrupted by a clear increase in the amplitude of the expiratory EMG, and air sac pressure displayed the characteristic song-like (but reduced in amplitude) morphology and temporal pattern (see Fig. 1b and Supplementary Fig. S5). The inspection of the infrared recordings of the birds confirmed that their eyes were



**Fig. 2** Statistical test to determine song-like night-sleep activity. **a** UMAP bidimensional projection allows an algorithmic clustering of song pressure patterns (bird 1). The numbers indicate the relative frequency of each cluster out of a total of 17,175 pressure patterns used (see Table 1). Examples of pressure patterns from each cluster are overlaid and displayed as thin lines (100 patterns per cluster). The arrow and color indicate the cluster from which these patterns were obtained. **b** Intra and inter clusters pairwise correlation distributions for song pressure patterns. Distribution of the correlation obtained between selected night-sleep pressure fluctuations and its most similar song pressure pattern (see text for details)

closed, and they were in a sleep-like posture (drooping head or head tucked in plumage). Furthermore, prior, and after these SLA events, respiratory rate and shallow breaths were typical of sleep.

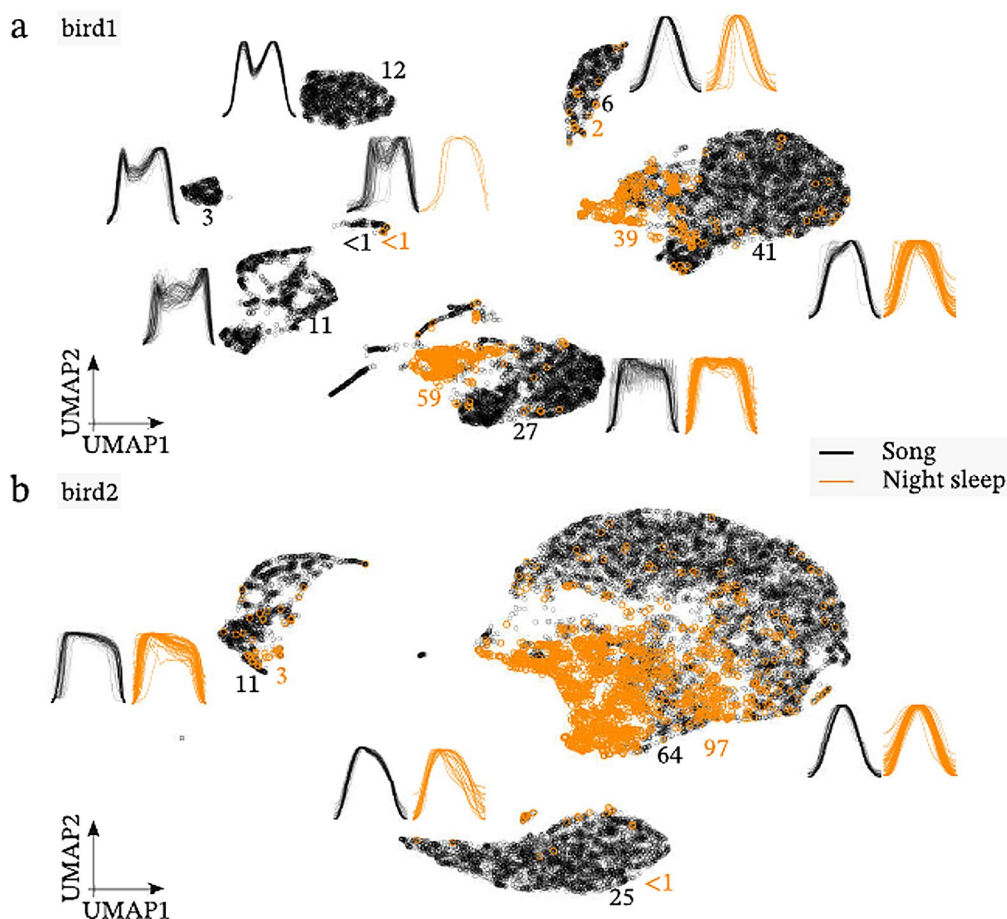
To determine whether a selected night-sleep pressure fluctuation (between two consecutive inspirations) could be associated with a song pressure pattern and thus accepted as SLA, a statistical test was performed. This analysis was performed as follows. First, we conducted an automatic clustering of air sac pressure patterns during song (awake)

as shown in Fig. 2a. Each song pattern was resampled to have 1,000 points, normalized to  $[-1,1]$  (see Methods), and a two-dimensional UMAP projection was obtained. The HDBSCAN method, with a minimum cluster size of 2% of the dataset size, was used to cluster the respiratory patterns algorithmically (see Methods). Next, we computed the pairwise Pearson cross-correlation coefficients for pressure patterns within (intra-clusters) and across clusters (inter-clusters). Figure 2b shows an example of the distributions obtained for one of the birds. The correlation coefficient was used as the statistic to determine whether two song pressure patterns belong to the same syllable type. The inter-cluster histogram describes the distribution of the statistic under the null hypothesis (that the patterns do not belong to the same type), while the intra-cluster histogram describes the distribution under the alternative hypothesis. A critical correlation coefficient was defined for a 5% significance level, and the significance and the power of the test were numerically computed as the area under the histograms (Table 1). With this setting, defined entirely by the awake song-pressure patterns, we performed the test to the selected night-sleep pressure fluctuations. Each one was resampled and normalized in the same way as the song pressure patterns. Its highest-correlation day-time song pressure pattern was identified, and if the correlation coefficient exceeded the 5% threshold, the pressure pulse was accepted as song-like. Even with this stringent criterion, the majority of identified sleep pressure fluctuations passed the test (Table 1) and thus were accepted as SLA.

During daytime song, each pressure pattern is repeated, but the relative frequencies of their occurrence differ. These relative frequencies describe how many syllables are repeated in a phrase (or trill) and how often that phrase is sang. The question arises whether these relative frequencies are preserved in SLA events. To answer this question, we performed a UMAP projection of the song pressure patterns, now including SLA pressure patterns. In Fig. 3 we show the result for two of the birds. Replayed patterns are mapped within song clusters, providing an independent quantification of their similarity. Interestingly, the relative number of patterns in each cluster (number of patterns divided by the total) is clearly not the same for song and night-sleep

**Table 1** Statistical test to determine respiratory night sleep song-like activity. The number of nights recorded is displayed in Supplementary Table S1

| Bird ID                | #Song syllables | #Song clusters | Critical correlation | Significance (%) | Power (%) | #SLA candidates | #SLA accepted |
|------------------------|-----------------|----------------|----------------------|------------------|-----------|-----------------|---------------|
| CaFF073-RoVio (bird1)  | 17,175          | 6              | 0.95                 | 5                | 84        | 893             | 854           |
| CaFF909-NaRo (bird2)   | 15,855          | 3              | 0.97                 | 5                | 73        | 2112            | 1947          |
| CaFF028-RoNe (bird3)   | 10,214          | 8              | 0.97                 | 5                | 54        | 40              | 29            |
| CaFF0481-AmaVe (bird4) | 5474            | 3              | 0.95                 | 5                | 68        | 159             | 150           |
| CaFF016-VioVio (bird5) | 19,270          | 5              | 0.93                 | 5                | 91        | 400             | 346           |



**Fig. 3** Identification of replayed pressure patterns. UMAP bidimensional projection of song and night sleep song-like pressure patterns for bird 1 (**a**) and bird 2 (**b**). The numbers indicate the relative frequency of each cluster for song (black) and night sleep (orange), a

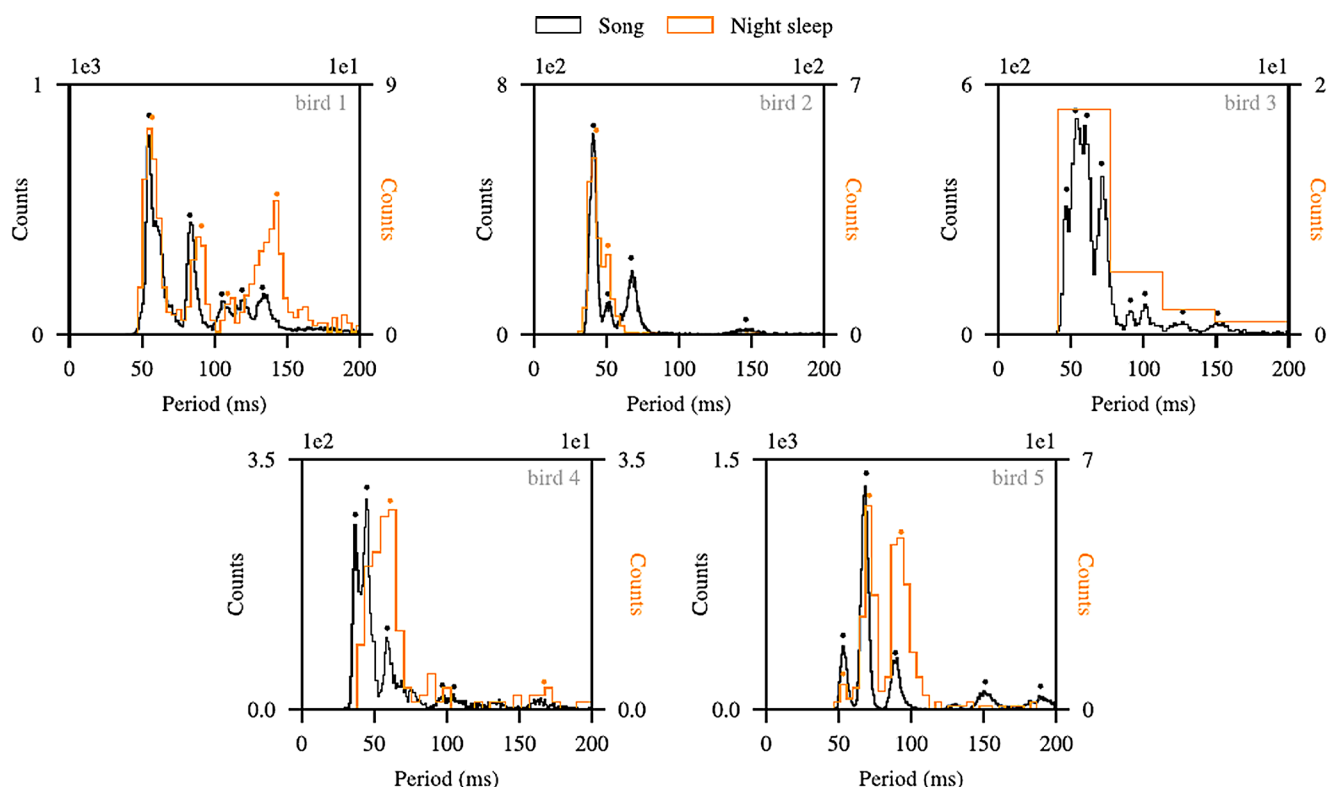
2% minimum size for clustering is used (see Methods). Examples of pressure patterns from each cluster are overlaid and displayed as thin lines for song (black) and night-sleep (orange)

activation (see Fig. 3). Relative frequencies of pressure patterns during daytime song are not preserved during SLA.

Canary song has a precise and rich rhythmic composition (Trevisan et al. 2006; Boari et al. 2022). The coarse temporal pattern of the song is largely established by the respiratory system. In Fig. 4 we show the comparison of the duration (or period) of the pressure patterns during song and night-time SLA. Song period presents multimodal distributions, with marked peaks which are mostly associated with single pressure pattern types. During night-time SLA, the occurrence of syllable types differs from that during day-time song. For instance, occurrence of 70 ms and 150 ms syllables of bird 2 are highly reduced, as was shown in Fig. 3b, in which two of the three clusters had only few replayed patterns. On the other hand, relative frequency of other syllables is increased, as can be seen in 140 ms syllables of bird 1, 50 ms syllables of bird 2, or 90 ms syllables of bird 5. We found that these syllables are the most frequently used at the beginning of daytime song performances and that this order is also preserved in replay events. Since

night-time SLA consisted of song-like events that were significantly shorter than daytime songs, these syllables end up overrepresented during night-sleep replays. The durations of the song and SLA events (i.e. the whole sequence, which includes many syllables) were found to be  $(5.6 \pm 0.1) s$  and  $(1.49 \pm 0.06) s$ , respectively (mean and standard error across all birds, see Supplementary Fig. S6, Welch's one-tailed t-test  $P < 0.001$ ).

Additionally, rhythm, as determined by pressure syllabic rates, is one of the features that characterizes a bird's song. Does replay activity preserve those individual features? Do song and night-sleep syllables have similar duration? We find that, aside from some relative frequency differences, song and night sleep period distributions show marked similarities (<10% difference between peaks, see Supplementary Methods) and individual characteristics can be recognized in the structure of night sleep distributions (Fig. 4).



**Fig. 4** Comparison of rhythm between song and night sleep events. Distribution of respiratory periods during song (black) and night sleep (orange) for each bird. Dots indicate histogram peaks persistent to bin

### Replay in syringeal muscles

We then investigated whether respiratory SLA occurs together with SLA of syringeal muscles. We conducted experiments measuring air sac pressure and EMG activity of the ventral syringeal (vS) muscle. The vS muscle (on both the right and left side of the syrinx) inserts on the second bronchial cartilage, so its contraction stretches the labia. Figure 5a shows an example of the activity recorded during song production, where each air sac pressure respiratory pattern of song also shows a characteristic activation pattern in the vS. During each occurrence of respiratory SLA simultaneous SLA in the syringeal muscle occurred (Figs. 5b and 1,830 replay patterns from  $n=2$  birds). This demonstrates that complete peripheral replay activation occurs in adult male canaries during night sleep. We never observed respiratory SLA without syringeal SLA. However, we also found many instances of clear SLA of the syringeal muscle without respiratory SLA. During these episodes, air sac pressure exhibited the slow, normal respiration cycle of sleep (Fig. 5c). In some cases, small air sac pressure fluctuations in phase with the syllabic reactivation of the muscle were observed, likely due to changes in airflow resistance when the syringeal muscles contract. These fluctuations

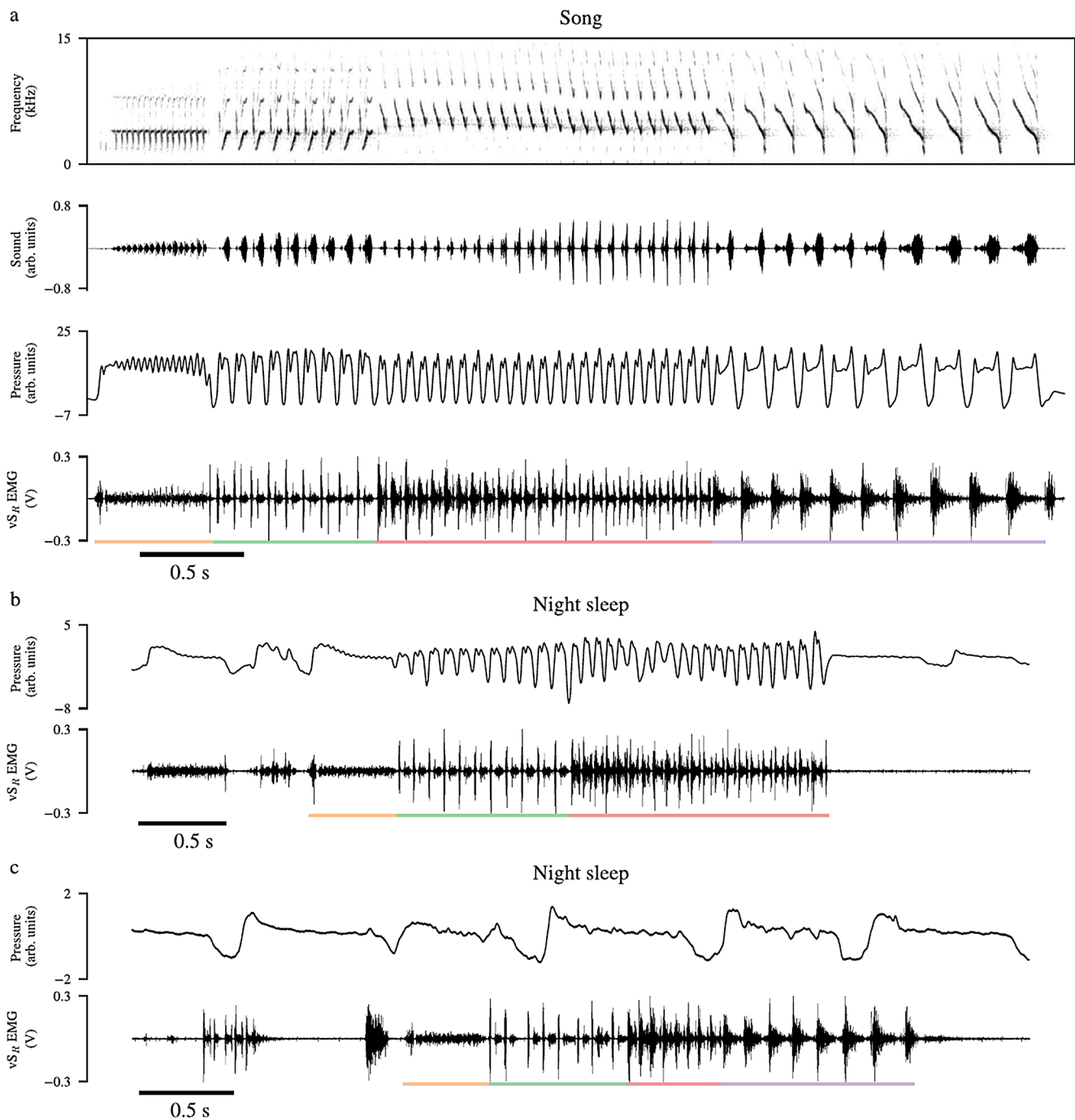
size (see Supplementary Methods and Supplementary Fig. S7). Note that the vertical scales for song and night sleep may differ due to differences in sample size

were clearly of smaller amplitude than the ones observed during respiratory SLA.

Previous studies in zebra finches have found replay activity of syringeal muscles without song-like activity in the respiratory system (Young et al. 2017). This raises questions about the frequency of occurrence of syringeal and complete replay events and their distribution throughout the night. To address these questions, we conducted an algorithmic search for vS EMG song-like activity during sleep. We selected vS EMG envelope templates during song production (see Supplementary Fig. S2). These templates were used to identify highly correlated segments during sleep. Applying a stringent statistical test, song-like activity was determined (see Methods and Supplementary Table S3). Moreover, this analysis allows the identification of replayed syllables during night sleep, as displayed in Fig. 5.

Respiratory SLA tended to occur more frequently later during the night (Fig. 6a) than syringeal SLA (Fig. 6b). In the two birds from which we recorded syringeal EMG, complete peripheral replay patterns (i.e., simultaneous syringeal and respiratory SLA) are predominantly found in the second half of the night, while syringeal SLA alone occurs almost continuously throughout the night. Analyzing vS EMG activity recorded during the day, no silent song-like vS EMG activity was detected (see Supplementary Fig.



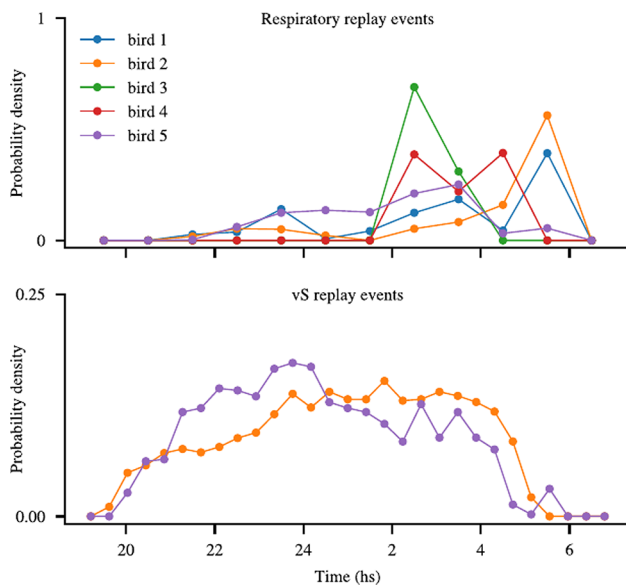


**Fig. 5** Complete and partial peripheral reactivation during night sleep (bird 5). **a** Syringeal muscle and respiratory activity during song. Color bars indicate the time interval of each syllable and outline the pressure and EMG patterns associated. The color code used is based on the vS EMG song templates selected for this bird (see Methods and Supple-

[S3](#) and Supplementary Table [S3](#)). We remark that we have not found SLA during the day. On the other hand, syringeal SLA is frequent throughout the night, with a mean of  $175 \pm 17$  and  $47 \pm 5$  vS EMG replay events per night, based on observations from 2 birds over 41 nights (see

Supplementary Fig. [S2](#)). **b** Respiratory replay events are always simultaneous to re-activation of syringeal muscles. **c** Syringeal muscles replay event without song-like respiratory activity. Peripheral song-like re-activation during night sleep allows the identification of replayed syllables

Supplementary Table [S3](#)). Interestingly, only about 1% of these events also involved respiratory SLA. An interesting difference between partial and complete peripheral SLA events is that syringeal SLA alone events seem to be more variable. As illustrated in Figs. [1](#) and [5](#), complete peripheral



**Fig. 6** Probability of occurrence of sleep replay event throughout the night. Complete replay events (upper panel), and syringeal replay events (lower panel). Sample numbers are shown in Table 1 and Supplementary Table S3

reactivation preserves the syllabic order in which daytime songs begin. This feature often does not occur in syringeal SLA alone events, in which replay activity may commence with syllables that never initiate a daytime song sequence.

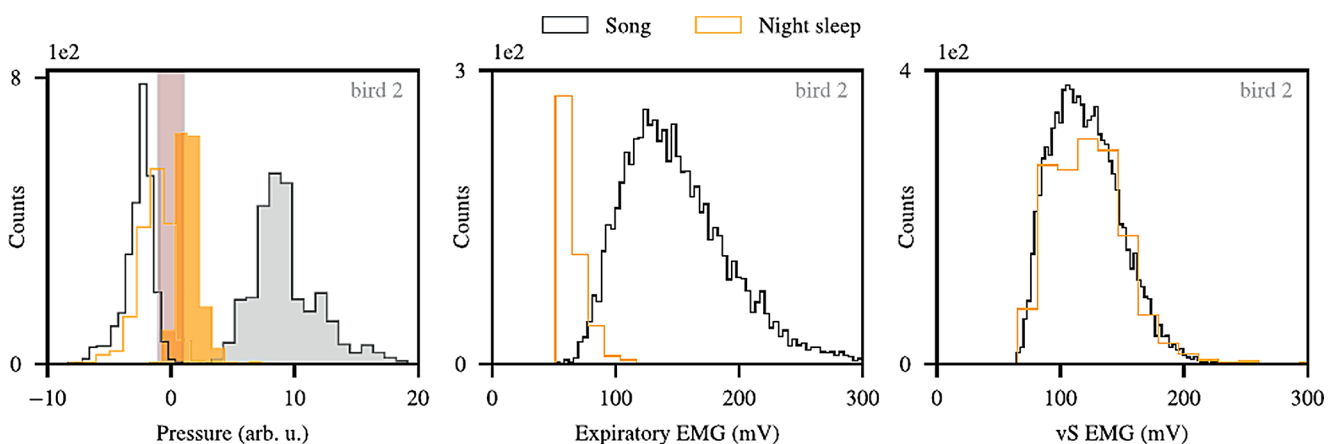
We also compared the amplitude of air sac pressure, expiratory EMG, and vS EMG during song and SLA. Scaling air sac pressure so that normal breathing oscillates between  $-1$  and  $1$  (see Supplementary Methods), we found that the peak-to-peak amplitude during song ( $19.8 \pm 0.1$  arb. u.) was reduced by approximately 75% during SLA at night ( $4.71 \pm 0.08$  arb. u., Welch's one-tailed t-test,  $P < 0.001$ ). Interestingly, this amplitude reduction is mainly due to a decrease in the expiratory phase (see Figs. 1, 5 and 7). A

similar result was observed for syllabic expiratory EMG amplitude, calculated as the maximum value (see Supplementary Table S4). For instance, the song expiratory EMG amplitude for bird 2 was  $150.5 \pm 0.6$  mV, while it was  $65.4 \pm 0.5$  mV during night sleep replay events (Welch's one-tailed t-test,  $P < 0.001$ , see Fig. 7). Conversely, the vS EMG amplitude during song and replay events, while significantly different, was much more similar (see Supplementary Table S4). For bird 2 it was  $120.9 \pm 0.3$  mV for song and  $125 \pm 1$  mV for replayed syllables (Welch's two-tailed t-test,  $P < 0.001$ ).

## Discussion

In zebra finches, spontaneous neuronal SLA during sleep has been documented throughout the song control circuitry, specifically in HVC (used as a proper name) (Hahnloser et al. 2006), the robust nucleus of the arcopallium (RA) (Dave and Margoliash 2000; Shank and Margoliash 2009; Elmaleh et al. 2023) and Area X (Yanagihara and Hessler 2012). This replay activity in the forebrain circuitry for song production leads to activation of the syringeal muscles, while respiratory muscles are not activated in song-like fashion (Young et al. 2017). Similarly, SLA patterns of syringeal muscles without concurrent SLA of respiratory muscles during sleep have also been observed in two suboscine species (Döppler et al. 2021), for which song production does not require forebrain circuitry (Gahr 2000; Nieder and Mooney 2020). Here we show that spontaneous SLA of syringeal muscles also occurs in sleeping adult male canaries. In contrast to these other species however, canaries also display SLA of respiratory muscles during the later sleep phase.

During SLA of respiratory muscles, temporal patterns of expiratory air sac pressure pulses and mini-breaths match



**Fig. 7** Amplitude comparison during song and night-time SLA events. Left panel shows the minimum and maximum (filled histograms) air sac pressure for each syllable. The shaded region shows normal breath-

ing amplitude. Expiratory EMG and vS EMG syllabic amplitude, computed as the maximum value, are shown in the middle and right panels, respectively

those of day-time song, but the amplitude, specifically that of expiratory pulses, is reduced. A decrease in air sac pressure peak-to-peak amplitude of approximately 75% lowers the aerodynamic drive sufficiently to prevent sound production. Sub-atmospheric air sac pressure pulses driving mini-breaths are not reduced to the same extent. SLA in the respiratory system is always accompanied by simultaneous reactivation of syringeal muscles, while syringeal SLA frequently occurs without respiratory activation.

Why do canaries occasionally also activate respiratory muscles during sleep, while all other investigated species do not? First, we will address this question in terms of central mechanisms. As pointed out above, SLA occurs in the main motor pathway for song production. HVC and the lateral magnocellular nucleus of the anterior nidopallium (LMAN) (Tian et al. 2023) SLA may drive SLA of RA neurons, which in turn activate the motor nucleus of syringeal muscles (nXII). RA also projects to the pacemaker circuitry for respiration (nuclei retroambigualis and parambigualis, RAM/PAM). One possibility is that inhibition at the level of RAM and/or PAM typically prevents SLA of the respiratory system. However, in canaries, this inhibition may be reduced later at night, thus allowing partial activation of the respiratory system. Interestingly, the amplitude of expiratory pressures appears to be reduced uniformly during all SLA events. This suggests that the downscaling of inhibition occurs as a single step predominantly in the activation of expiratory motor neurons. This partial inhibition may lead to activation of a subset of the expiratory muscle motor units typically activated during song, thus leading to reduced amplitude in expiratory pressure. Nevertheless, the temporal pattern of song is present and overrides the normal quiet respiratory tempo of sleep. The cytoarchitecture of the respiratory pacemaker and its dynamics may account for the emergence of the correct temporal pattern despite the reduction in recruitment of muscle motor units. Another possibility may be related to different stages of sleep. Studies in zebra finch sleep have found a change in the proportion of slow-wave sleep (SWS) and rapid-eye-movement sleep (REM) (Low et al. 2008; Yeganegi and Ondracek 2023), with an increase in REM sleep at the end of the night. Our observed increase in complete peripheral SLA at the end of the night could be related with this observation.

In zebra finches, SLA in HVC and RA does not seem to be initiated by thalamic input (Elmaleh et al. 2021), whereas day-time singing is driven by such input (Moll et al. 2023). Presumably, the thalamus receives upstream information from the respiratory pacemaker circuit of the brainstem (Ashmore et al. 2008) and thus coordinates the onset of song with respiratory state. If this input to the forebrain motor production circuitry is not replayed during sleep, the respiratory premotor dynamics may not allow full activation for

song production. Partial reduction in sleep state induced inhibition in canaries during the latter part of the sleep phase may be related to preparation for the awake state. Why this is not occurring in zebra finches and suboscines is not known.

A second viewpoint of the above question is whether activation of the respiratory system during sleep in canaries serves a specific function. It is widely assumed that night-time replay of motor activity enhances motor memory and maintenance of motor patterns (Derégnaucourt et al. 2005). While this explains SLA in the central motor circuitry, the fact that syringeal muscles are activated remains unexplained. Peripheral activation of the syrinx could simply be a byproduct of the cranial control of its muscles and not serve a particular function. However, the fact that in canaries, respiratory muscles are also activated, weakens this viewpoint and, considering the above mechanistic interpretation, suggests that peripheral motor behavior may be functionally relevant. Activation of muscles generates somatosensory feedback, which could be critical for maintaining day-time stereotyped motor performance (e.g., Young et al. 2017). Alternatively, activation of muscles during the night might serve in conserving muscle performance, as specific gene expression patterns for myosin composition and proteins involved in  $\text{Ca}^{2+}$  cycling are, in part, regulated by muscle use (e.g., Frontera and Ochala 2015; Bolaños and Calderón 2022; Hoh 2023). Further studies are needed to test this hypothesis – while the superfast kinetics of syringeal muscles may require avoidance of disuse over long periods, a similar requirement for the much slower respiratory muscles seems less likely.

The difference in peripheral SLA between zebra finches and canaries may be attributed to their distinct singing behavior. In zebra finches, SLA during sleep is characterized by activation events that differs from the highly stereotyped motor sequences of day-time song. Song motifs are often incomplete, syllable sequence is altered, and left-right control is not coordinated (Young et al. 2017; Elmaleh et al. 2021). In canaries, syllable structures during complete ( $vS$  and pressure) peripheral SLA does not appear to be more variable than during daytime singing, although the duration of SLA sequences is shorter than that of songs and the frequency of occurrence of syllable types is different. Complete peripheral SLA in canaries preserves the syllabic order in which songs begin and largely match daytime song performance. However, syringeal SLA alone events are more variable, often starting with syllables that never initiate day-time songs and displaying replay of individual phrases.

Previous studies have found a slowing of rhythm and shorter SLA pattern duration compared to daytime song (Young et al. 2017; Döppler et al. 2021), potentially influenced by factors such as differences in melatonin levels (Derégnaucourt et al. 2012) and lower brain temperature

(Long and Fee 2008; Goldin et al. 2013). In canaries, however, the differences in pattern duration between song and SLA appear to be more subtle. Experiments in a suboscine species revealed that the burst repetition rate during sleep was slower during the replay of the bird's typical song, but not during the replay of the trill (Döppler et al. 2021), the latter also involving a partial behavioral display. Since canary song predominantly consists of trill-like vocalizations, these findings suggest that trill production dynamics may be less affected by the sleeping state compared to other songs that do not involve the repetition of syllables.

This difference in replayed SLA between zebra finches and canaries suggests potential differences in how this activation contributes to the maintenance of the song motor program. In canaries, song syllable repertoires are substantially greater than in the zebra finch, and variable phrase sequences generate greater complexity of temporal structure. Furthermore, adult zebra finches sing a stereotyped song sequence after song ontogeny is completed, while canaries undergo annual periods of plasticity during which new syllables can be added to the repertoire and phrase sequences can change (e.g., Prather et al. 2008; Lehongre and Del Negro 2009). Perhaps these differences in song organization and plasticity are reflected in the underlying dynamics of the neural circuitry, thus causing the observed differences in SLA expression in the peripheral motor systems for song production. Perhaps occasional respiratory SLA in addition to syringeal SLA enhances the consolidation of restructured motor patterns, which include complex, rapid respiratory motor actions. In contrast, zebra finches do not change their syllable sequence after ontogeny and suboscines sing comparatively simple, stereotyped songs that develop innately (e.g., Nowicki and Searcy 2014). Studies on other open-ended learners could test whether the peripheral SLA activation patterns of canaries are linked to adult plasticity.

Measuring and decoding ensemble dynamics during sleep replay remains a key challenge in any system. The complete peripheral reactivation of the respiratory system and the syrinx offers unique and detailed insight into central replay activity. This comprehensive peripheral reactivation during sleep paves the way for robust and interpretable decoding of motor instructions for a learned, complex behavior. The current study also stresses the need for comparative data sets. The differences and similarities in SLA in the context of variable song organization between different species allow insights into the complex neural dynamics of song motor control and sensorimotor mechanisms. Here we showed differences in SLA between the zebra finch and canary, whose interpretation allowed posing of different testable hypotheses. More comparative work is necessary for illumination of how peripheral SLA contributes to development and maintenance of the song motor program.

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**Data availability** Data will be available under request.

## Declarations

**Competing interests** The authors declare no competing interests.

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